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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
14 October 1999 (14.10.99)

(54) Title: PEPTIDES FOR INHIBITION OF APOPTOSIS AND PHARMACEUTICAL COMPOSITIONS CONTAINING THEM

(57) Abstract

The present invention relates to an isolated and purified peptide of the RY domain having an amino acid sequence of general formula (I) comprising a sequence of the following amino acids: $X_1 - X_2 - X_3 - X_4 - X_1 - X_4 - X_4 - X_3 - X_1$, X_1 = Phe, Tyr, or any amino acid having a substituted aromatic residue; X_2 = Glu, Asp, Ser, or any amino acid having a $-(CH_2)_n-COO$ residue, wherein n = 0-3; X_3 = Asp, Thr, any aliphatic amino acid, or any of amino acids X_4; and X_4 = Arg, Lys, or any amino acid having a $-(CH_2)_n-NH_3^+$ residue, or a $-(CH_2)_n-NH-C(NH_3^+)NH_2$ residue wherein n = 0-4; as well as functional equivalents thereof. The invention relates also to pharmaceutical compositions comprising a compound of general formula (I), the use of said RY peptide and of said pharmaceutical composition in the preparation of a medicament and in methods for the treatment of disorders of inappropriate activation of apoptosis; for increasing the number of viable cells in a biological tissue; and in a method for the enhancement for the survival of biological cells. The invention also relates to a method for the preparation of a RY-peptide of general formula (I). Furthermore, the invention also relates to an in vitro assay system for the regulation of cell death by the Bcl-2 family of test compounds (as hereinbefore defined).

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/IL 99/00096

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: As far as claims 13-15 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[] The additional search fees were accompanied by the applicant's protest.

[] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IL 99/00096

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9805777 A	12-02-1998	EP 0917574 A	26-05-1999
WO 9735971 A	02-10-1997	AU 2143997 A	17-10-1997
		CA 2250207 A	02-10-1997
		EP 0932674 A	04-08-1999
WO 9629403 A	26-09-1996	US 5789389 A	04-08-1998

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IL 99/00096

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 35971 A (ADAMS JERRY MCKEE ;HOLMGREEN SHAUN P (AU); CORY SUZANNE (AU); GIBS) 2 October 1997 (1997-10-02) Seq ID 7 and 9 page 2, line 27 - page 3, line 5 page 5, line 25 - page 6, line 5; claims; examples	1,5,6, 10-15,21
X	WO 96 29403 A (UNIV PENNSYLVANIA) 26 September 1996 (1996-09-26) Seq ID 6, 8 and 10 claims; examples	1,5,6, 10-15,21

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int[illegible]lication No
PCT/IL[illegible]/00096

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 A61K38/17 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S.M. MUCHMORE ET AL.: "X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death" NATURE, vol. 381, 23 May 1996 (1996-05-23), pages 335-341, XP002111426 LONDON GB cited in the application page 339, left-hand column, paragraph 2 - page 340, left-hand column, paragraph 1 ---	1
X	WO 98 05777 A (DANA FARBER CANCER INST INC) 12 February 1998 (1998-02-12) SEq ID 2 page 28, line 12 - page 30, line 34; claims; examples --- -/--	1,5,6, 10-15,21

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 August 1999	19/08/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fuhr, C

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Form PCT/ISA/210 (second sheet) (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
8243		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IL99/00096	16/02/1999	24/02/1998
International Patent Classification (IPC) or national classification and IPC C07K14/47		
Applicant NST NEUROSURVIVAL TECHNOLOGIES LTD. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

 ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

 These annexes consist of a total of 25 sheets.

3. This report contains indications relating to the following items:

 - I ☒ Basis of the report
 - II ☐ Priority
 - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
 - IV ☒ Lack of unity of invention
 - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
 - VI ☐ Certain documents cited
 - VII ☐ Certain defects in the international application
 - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
16/08/1999	14.07.00
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Paresce. D Telephone No. +49 89 2399 8995

Form PCT/IPEA/409 (cover sheet) (January 1994)

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PATENT COOPERATION TREATY

MAY -07- 23

23-07-2000

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

HESS, Yitzhak et al.
Yitzhak, Hess & Partners
P.O.Box 6451
TEL-AVIV 61063
ISRAEL

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	14.07.00
Applicant's or agent's file reference 8243	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IL99/00096	16/02/1999	24/02/1998

Applicant
NST NEUROSURVIVAL TECHNOLOGIES LTD. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Faux, K Tel. +49 89 2399-8062

Form PCT/IPEA/416 (July 1992)

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL99/00096

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-5,10,11,16,22	as originally filed			
6-9,12-15,17-21	as received on	30/05/2000	with letter of	28/05/2000

Claims, No.:

22-24	as received on	30/03/2000	with letter of	27/03/2000
1-21	as received on	30/05/2000	with letter of	28/05/2000

Drawings, sheets:

1/9,4/9,5/9	as originally filed			
2/9,3/9,6/9-9/9	as received on	30/05/2000	with letter of	28/05/2000

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☒ restricted the claims.

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☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☒ complied with.

☐ not complied with for the following reasons:

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-24
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-24
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-24
	No:	Claims	

2. Citations and explanations

see separate sheet

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VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

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Re Item I
Basis of the report

This international preliminary examination report will be based on amended claims 1-21 submitted by the Applicant with a letter dated 28.05.00 and claims 22-24 submitted by the Applicant with a letter dated 27.03.00. The amended set of claims is supported by the original disclosure and therefore complies with the requirements of Article 34(2)(b) PCT.

Re Item IV
Lack of unity of invention

In Form PCT/IPEA/405, sent on 01.03.00, the IPEA found that the claims as filed lacked unity within the meaning of Rule 13.1 PCT. The IPEA found that the claims as filed related to two different inventions. The separate inventions/groups of invention are:

1) Claims 1-21 are directed to isolated peptides comprising an amino acid sequence of the general formula I, defined in claim 1 of the present application, as well as functional equivalents of said peptides. This includes peptides **comprising** said amino acid sequence as well as short peptides **having** specific sequences of general formula I. These claims are further directed to pharmaceutical compositions comprising said peptides or functional equivalents of said peptides, methods of use of said peptides, and methods of preparation of said peptides.

2) Claims 22-25 are directed to an in vitro assay system for the regulation of cell death by the Bcl-2 family of test compounds.

In reply to the non-unity objection raised by the IPEA, the Applicant has amended claims 22-25. These claims now refer to isolated peptides of the RY domain according to claims 1 to 9. The IPEA considers, therefore, that the amended set of claims now fulfill the requirements of Rule 13.1 PCT and relate to a single invention.

Re Item V

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Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1) The documents mentioned in this communication are numbered as in the search report, i.e. D1 corresponds to the first document of the search report.

2) D1 discloses the X-ray and NMR structure of human Bcl-X_L, an inhibitor of programmed cell death. D1 shows a sequence alignment of Bcl-2 family members: Bcl-X_L, Bcl-2, Bak, Bax, Mcl-1, Ced-9. Both Bcl-X_L and Bcl-2 contain the "RY domain". Both Bcl-X_L and Bcl-2 have sequences comprising an amino acid sequence of the general formula I, defined in claim 1 of the present application (see figure 1, d, amino acids 97-105). This domain overlaps with a homology domain BH3 identified in D1 as a region of homology among Bcl-2 proteins that was found to be essential for the activity of the death-promoting proteins. D1 discloses that the three homology regions, BH1-BH3, are in close spatial proximity and form an elongated hydrophobic cleft in Bcl-X_L. D1 proposes that this cleft may be the site of interaction with death-promoting proteins such as Bax and Bak (see p. 339).

D2 discloses a novel isoform of the Bcl-x family of proteins called Bcl-xγ. This peptide is expressed in T-lymphocytes and is associated with resistance to apoptosis. The amino acid sequence of Bcl-xγ is shown in SEQ ID NO:2. This protein comprises an amino acid sequence of the general formula I, defined in claim 1 of the present application (see SEQ ID NO:2, p. 84). D2 discloses an isolated peptide, that has anti-apoptotic activity, comprising a specific domain of Bcl-xγ (the γ domain). D2 further describes methods of use of said proteins as well as diagnostic assays using said proteins (see claims).

D3 discloses another member of the Bcl-2 family of proteins called Bcl-w. The amino acid sequence of Bcl-w is shown in SEQ ID NO:7 or SEQ ID NO:9. This protein comprises an amino acid sequence of the general formula I, defined in claim 1 of the present application (see SEQ ID NO:7 or SEQ ID NO:9). D3 also discloses derivatives or fragments of said protein (see p.5). D3 further describes methods of use of said proteins in therapy, diagnostic assays, antibody generation

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and as a screening tool for therapeutic molecules capable of modulating physiological cell death or survival and/or modulating cell cycle entry (see abstract and claims).

The present invention involves the isolation of a specific protein domain called the "RY domain" which is similar among death inhibitors (Bcl-2, Bcl-xL, Bcl-w) but markedly different in death inducers (Bax, Bak) (present application, p. 5-6). The isolated peptide of said RY domain was found to have a death inhibiting activity. The IPEA is of the opinion that the identification of the RY sequence and the characterization of its anti-apoptotic activity is both novel and inventive. D1 discloses several conserved regions in the Bcl-2 proteins and in particular mentions the BH3 domain which is identified in D1 as a region of homology among Bcl-2 proteins that was found to be essential for the activity of the death-promoting proteins. D1 does not, however, disclose the RY sequence as a distinct sequence. From the teachings of D1 it would not be expected that a domain adjacent to the BH3 domain, which is implicated in death promoting activity, would instead have death inhibiting activity. Furthermore, the use of the isolated peptide of the RY domain of the present application presents several advantages over the use of other death inhibitory proteins disclosed in the prior art. The present invention provides a small protein fragment which can mimic the activity of the whole protein. There are many advantages to the use of a small peptide as a drug candidate rather than the full-length protein (for example, rate of degradation or immunogenicity). Therefore, the IPEA is of the opinion that isolated peptides having the amino acid sequence of general formula I as defined in claim 1 have not been made available to the public by any of the available prior art documents and can therefore be regarded as novel. Furthermore, the IPEA considers that the subject-matter of claims 1-24 cannot be derived from the available prior art in an obvious manner and therefore complies with the requirements of Article 33(3) PCT.

VIII. Certain observations on the international application

1) Clarity: Article 6 PCT

Claims 1, 10 lack clarity due to the expression "functional equivalents thereof".

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This term is vague and unclear and leaves the reader in doubt as to the meaning of the technical features to which it refers, thereby rendering the definition of the subject-matter of said claim unclear (Article 6 PCT).

Claims 21, 24 are not clear. As is stated in Rule 6 (2a) PCT, claims shall not, except where absolutely necessary, rely, in respect of the technical features of the invention, on references to the description or drawings. In particular, they shall not rely on such references as: "as described in part...of the description" or "substantially as herein described with reference to the examples".

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PEPTIDES FOR INHIBITION OF APOPTOSIS AND PHARMACEUTICAL COMPOSITIONS CONTAINING THEM

The present invention relates to the field of cell death, and more particularly, to apoptosis. The novel peptides and the compositions comprising said peptides are useful in inhibiting cell death. Therefore, they are potentially useful in treating disorders of inappropriate activation of cell death, such as neurodegenerative disorders, cerebral strokes, myocardial infarctions, etc.

Apoptosis is an intrinsic cell self-destruction or "suicide" program. In response to a triggering stimulus, cells undergo a highly characteristic cascade of events of cell shrinkage, blebbing of cell membranes, chromatin condensation and fragmentation, culminating in cell conversion to clusters of membrane-bound particles (apoptotic bodies), which are thereafter engulfed by macrophages (Boobis AR, et al. Trends Pharmacol. Sci. 10:275-280, 1989; Bursch W, et al. Trends Pharmacol. Sci. 13:245-151, 1992.).

Normally, apoptosis plays important physiological roles, among others in the development of the central nervous system (Merry DE, et al. Development 10: 301-311, 1994.) However, it is now known that "inappropriate" activation of this death program also plays a critical part in the pathogenesis of numerous disorders, e.g. AIDS, ischemic injuries such as cerebral strokes or myocardial infarctions, and neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease or amyotrophic lateral sclerosis (ALS) (Ziv I, et al. Neuosci Lett. 170: 136-140, 1994; Ziv I, et al. J. Neural. Transm. 49 (supp): 69-75, 1997; Thompson CB. Science 267:1456-1461.). The etiologies of the latter frequent and progressive neurological disorders are unknown. Thus, there are no known therapeutic measures capable of affecting the downhill course of the neuro-degenerative process. However, the substantiation of the role of apoptosis in the neuronal death in these disorders now delineates a novel window for therapeutic interventions, aimed to inhibit the final common biochemical pathway of the apoptotic process, upon which the various triggers of the death program converge.

The Bcl-2 family of proteins is a major system controlling this final common pathway. This growing family of proteins includes death-inhibitory members (Bcl-2, Bcl-xL, Bcl-w, Ced-9, Mcl-1, A1) as well as death inducers (Bax, Bak, Bcl-xS, Bad, Bik, Bid, Hrk) (Kroemer G. Nat. Med. 3:614-620, 1997; Reed JC. Nature 387:773-776, 1997.) This protein system has been shown to be a powerful regulator of cell death. Bcl-2 can protect cells from a wide array of insults, and can inhibit both apoptotic and necrotic modes of cell death (Shimizu S. Nature 374:811-813, 1995; Ziv I, et al. Apoptosis 2:149-155, 1997). On the other hand, transgenic Bcl-xL- knock-out mice manifest extensive apoptosis of neuronal tissues (Motoyama N, et al., Science 267:1506-1510), whereas neurons of Bax knock-out mice manifest resistance to apoptosis (Deckwerth TL, et al., Neuron 17:401-411, 1996). Clinical relevance of this protein system is reflected, among others, in reports of Bax upregulation following cerebral ischemia (McGibbon GA, et al. Brain Res. 750:223-234, 1997) and also in Alzheimer's disease brains and ALS spinal cord motor neurons (Su JH, et al. J. Neuropathol. Exp. Neuro. 56:86-93, 1997; Mu X, et al. Ann Neurol 40:379-386, 1996).

The members of the Bcl-2 family of proteins are strategically localized in the outer mitochondrial membrane, endoplasmic reticulum, nuclear envelope, and the cytosol (Kromer G. Nat Med 3:614-620, 1997; Reed JC Nature 387:773-776, 1997). Bcl-xL has a predominantly mitochondrial localization. Notably, Bcl-xL manifests high levels of expression in the central nervous system (Mizuguchi M, et al. Brain Res 712:281-286, 1996). Bax, a major death inducer, is predominantly cytosolic, but manifests redistribution to the mitochondria upon induction of apoptosis (Wolter KG, et al., J Cell Biol 139:1281-1292, 1997.

Amino acid sequence analysis of the Bcl-2 family yielded a focus on several regions within the proteins (Yin XM, et al. Nature 369:321-323, 1994; Sedlak TW, et al., Proc. Natl. Acad. Sci. USA, 92:7834-7838, 1995; Cheng EH, et al., Nature 379:554-556, 1996; Chittenden, T et al., EMBO J. 14:5589-5596, 1995; Hunter J. at al. J. Biol. Chem. 271:8521-8524, 1996; Wang K, et al., Genes Dev. 10:2859-1869, 1996). These regions are:

1. A hydrophobic C-terminal region, which serves for membrane

anchoring.

2. BH1 and BH2: Regions which are important for formation of a hydrophobic binding cleft, where protein-protein interactions take place.
3. BH3: The C-terminal half of the amphipathic Bcl-xL second helix, serves as part of the hydrophobic binding cleft. The homologous region in the death-inducing family members, serves as a ligand region, and is important for their protein-protein interactions with other proteins within the family.
4. A flexible, cytosol-exposed PEST-like region in Bcl-2 and Bcl-xL, which serves as a regulator region. It includes serine phosphorylation sites.
5. BH4: an N-terminal region, which serves to stabilize the three dimensional protein structure, as well as a critical docking region for several proteins, e.g., Raf-1, Bag-1 and Ced-4.

The mode of action of the Bcl-2 family proteins in the regulation of cell survival is largely unknown, though two major functions have been revealed:

1. Adaptor/Docking proteins: Bcl-2 and Bl-xL, by virtue of their membrane attachment with cystolic orientation, have been shown to act as important adaptor or docking proteins, pulling-out proteins from the cytosol, thus inactivating them or orienting them to interact with other membrane-bound proteins. Among these are the protein kinase Raf-1, calcineurin, R-Ras, H-Ras, the prion protein Pr-1, Bag-1, the p53-binding protein p53-BP2 and others (Kroemer G. Nat Med 3:614-620, 1997; Reed JC. Nature 387:773-776, 1997).
 Another important protein shown to be docked to Bcl-2 is the Apaf-1 protein. This death-inducing protein, homologous to the nematode Caenorhabditis-elegans protein CED-4, acts in mediating linkage between the Bcl- 2 system and the downstream cysteine proteases (caspases), which perform the execution phase of the death program. (Zou H et al., Cell 90:405-413, 1997; Yuan J., et al., Development 116:309-320, 1992; Chinnaiyan AM, et al., Science 275:1122-1126, 1997).
2. Formation of transmembrane pores and/or ionic channels, as

suggested by the similarity of the crystal structure of Bcl-xL and the structure of pore forming bacterial toxins, e.g. colicins and diphtheria toxin (Muchmore, et al., Nature 381:335-344;1996). Bcl-xL, Bcl-2 and Bax have all been shown to be capable of transmembrane ionic channel formation, the two formers only in acidic pH, whereas the latter also in physiological pH. (Minn AJ, et al. Nature 385:353-357, 1997; Schendel SL, et al. Proc. Natl. Acad. Sci. USA 94:5113-5118, 1997; Antonsson B et al., Science 277:370-372, 1997; Schlesinger PH, et al., Proc. Natl. Acad. Sci. USA 94:11357-11362, 1997).

These structure-function considerations and the localization of the Bcl-2 and Bcl-xL to the outer mitochondrial membrane, are in accordance with the emerging importance of the mitochondrial level in the apoptotic cascade (Zamzami N, et al. J. Exp Med 183:1533-1544, 1996). Disruption of the mitochondrial transmembrane potential has been shown to be an early event in apoptosis (Zamzami N, et al., J. Exp Med 181:1661-1672, 1995). Evidently, this derangement involves the opening of so-called mitochondrial permeability transition pores (PTP) (Zamzami N, et al., J Exp Med 182:367-377,1995). These are megachannels, which can be opened in response to numerous noxious stimuli and lead to redistribution of molecules of <1,500 daltons, thus disrupting mitochondrial membrane potential and associated mitochondrial functions (Zoratti M, et al., Biochim Biophys Acta 1241:139-176, 1995). Chemical inducers and inhibitors of the PTP have been shown to induce or inhibit apoptosis, respectively (Marchetti P, et al., Apoptosis 1:119-215, 1996; Zamzami N, et al., FEBS Letters 384:53-57, 1996). Moreover, during the apoptotic process, mitochondria have been shown to liberate at least three mediators of apoptosis: ceramide, cyctochrome c, and AIF; (apoptosis-inducing factor) a 50kDa protein with caspase-like activity (Marchetti P, et al., Apoptosis 1:119-125, 1996). Interestingly, all these mitochondrial-dependent steps in the apoptotic process have been effectively blocked by Bcl-2 (Zamzami N, et al., J Exp Med 182:367-377, 1995; Kluck RM, et al., Science 275:1132-1136, 1997; Susin S, et al., J Exp Med 184:1331-1342, 1996).

Specific importance is currently attached to the release

from the mitochondria of cytochrome c, which acts to activate the downstream apoptotic cascade, and can be liberated and exert its pro-apoptotic activity even in the absence of mitochondrial potential collapse (Bossy-Wetzel E, et al. EMBO J, 17:37-49, 1998; Li F, et al J Biol Chem, 272:30299-30305,1997).

The Bcl-2 system is therefore a powerful system in determining cell fate. For purposes of the development of novel diagnostics and therapeutics for modulation of cell death, it has therefore been desirable to identify specific regions or domains within these proteins which are, by themselves, capable of fulfilling at least part of the functions of the whole proteins. For example, the identification of agents capable of mimicking the apoptosis-inhibitory effects of Bcl-2 and Bcl-xL may be useful for the treatment of medical disorders associated with inappropriate activation of the death program, for example, neurodegenerative disorders, ischemic injury (cerebral stroke, myocardial infarction), AIDS, myelodysplastic syndromes, traumatic or toxic injuries.

The present invention relates to a novel protein domain which is useful in inhibiting cell death, and which has been identified and mapped to a short domain of 9 amino acids which is called hereinafter "RY domain" and which is mapped on the central portion of the relevant protein.

Said RY domain is:

1. Significantly conserved through evolution: Comparison of the Bcl-xL amino acid sequence from chicken, mouse, rat and human, revealed conservation of this motif between species. Conservation through evolution is usually considered to mark structural/functional importance.
2. Similar among death inhibitors: Comparison of the " RY domain" of the death inhibitors: Bcl-2, Bcl-xl and Bcl-w revealed marked similarity:

 Bcl-xL: Phe-Glu-Leu-Arg-Tyr-Arg-Arg-Ala-Phe (FELRYRRAF)

 Bcl-2: Phe-Ser-Arg-Arg-Tyr-Arg-Arg-Asp-Phe (FSRRYRRDF)

 Bcl-w: Phe-Glu-Thr-Arg-Phe-Arg-Arg-Thr-Phe (FETRFRRTF)
3. Located on the outer surface of the protein, as observed in the NMR and x-ray protein solution and crystal structures of Bcl-xL (Muchmore SW, et al., Nature 381:335-341, 1996;

Brookhaven Protein Data Bank, Accession No. 1LXL and 1MAZ, respectively). These structural data also reveal that this small region forms an almost closed loop within the protein structure, with the Phe1 and Phe9 in close spatial proximity, while the Arg side-chains face the outside of the protein (Figure 1).

4. Undergoing conformational change upon binding of Bcl-xL to a peptide from the BH3 region of a death inducer (Bak):
A systematic comparison has been performed of the structure of the free Bcl-xL (Muchmore SW, et al., Nature 381:335-341, 1996) and its structure upon binding to the BH3 peptide of Bak (Sattler M, et al., Science 275:983-986, 1997). In its unbound conformation, the second helix of the protein encompasses 15 amino acids (Ala84-Glu98). The RY domain in this conformation is the loop described above (Fig. 1.) However, upon binding of the BH3 peptide of Bak to the hydrophobic cleft of Bcl-xL, the RY domain undergoes a conformational change and becomes a part of the second helix, which now extends to residue Arg103 (Sattler M, et al., Science 275:983-986, 1997). Moreover, upon binding, several of the RY residues become engaged in interactions with the death-inducer peptide (i.e., Phe97,Arg100, Tyr101, Phe105).

5. Markedly different in cell death inhibitors vs. inducers:
Death inhibitors:
Bcl-xL: FELRYRRAF (Phe-Glu-Leu-Arg-Tyr-Arg-Arg-Ala-Phe)
Bcl-2: FSRRYRRDF (Phe-Ser-Arg-Arg-Tyr-Arg-Arg-Asp-Phe)
Bcl-w: FETRFRRTF (Phe-Glu-Thr-Arg-Phe-Arg-Arg-Thr-Phe)
Death inducers:
Bax: LDSNMEL (Leu-Asp-Ser-Asn-Met-Glu-Leu)
Bak: INRRYDSEF (Ile-Asn-Arg-Arg-Tyr-Asp-Ser-Glu-Phe)
It has been found that the peptide FELRYRRAF is a potent inhibitor of cell death.

The present invention thus consists in an isolated and purified peptide of the RY domain having an amino acid sequence of general formula I comprising a sequence of the following amino acids:

$$X_1 - X_2 - X_3 - X_4 - X_1 - X_4 - X_4 - X_3 - X_1 \qquad \text{I}$$

X_1 = Phe, Tyr, or any amino acid having a substituted

aromatic residue;

X_2 = Glu, Asp, Ser, or any amino acid having a $-(CH_2)_n-COO^-$ residue, wherein n = 0-3;

X_3 = Asp, Thr, any aliphatic amino acid, or any of amino acids X_4; and

X_4 = Arg, Lys, or any amino acid having a $-(CH_2)_n-NH_3^+$ residue, or a $-(CH_2)_n-NH-C(NH_3^+)NH_2$ residue wherein n = 0-4;

as well as functional equivalents thereof.

By "functional equivalents" is meant a compound possessing a biological activity or/and immunological characteristic similar to that of the RY domain of general formula I. Said term includes fragments, variants analogs, homologs and chemical derivatives possessing such activity or characteristic.

Any of the above amino acids may be either the D- or the L-isomer.

The amino acid residues may also be residues of suitable synthetic amino acids.

Methionine (Met) may be connected to the N-terminal of the sequence of general formula I and this sequence is also within the scope of the present invention.

In an advantageous sequence of general formula I the sequence

X_4 - X_1 - X_4 - X_4 stands for Arg - Tyr - Arg - Arg;

In a further embodiment said sequence is preceded by X_3 = Arg.

The substituted aromatic residue of X_1 is preferably Phenyl-$(CH_2)_n$-, wherein n = 0-3.

The aliphatic amino acid of X_3 is preferably selected among Leu, IIe, Ala, Gly and Val.

The preferred sequence according to the present invention is Phe-Glu-Leu-Arg-Tyr-Arg-Arg-Ala-Phe (FELRYRRAF).

Said sequence corresponds to residues 97 - 105 of the Bcl-xL protein.

Additional sequences which may be considered as suitable death inhibitors are:

Phe-Ser-Arg-Arg-Tyr-Arg-Arg-Asp-Phe (FSRRYRRDF)

Said sequence corresponds to residues 104 - 112 of the Bcl-2

protein.

Phe-Glu-Thr-Arg-Phe-Arg-Arg-Thr-Phe (FETRFRRTF).

Said sequence corresponds to residues 53 - 61 of the Bcl-w protein.

The present invention consists also in pharmaceutical compositions comprising as active ingredient of the RY- peptide, as defined above.

In a preferred embodiment the pharmaceutical composition comprises in addition to the RY-peptide a pharmaceutically acceptable carrier.

The pharmaceutical compositions may be, e.g. tablets, capsules, solutions, emulsions, etc. The carriers may be selected among any suitable components, e.g. solvents; emulgators; excipients; talc; flavors; colors; etc. The pharmaceutical composition may comprise, if desired, also other pharmaceutically active compounds.

The amount of the RY protein incorporated in the pharmaceutical composition may vary widely. The factors which have to be considered when determining the precise amount are known to those skilled in the art. Such factors include, inter alia, the pharmaceutical carrier being part of the composition, the route of administration being employed and the frequency with which the composition is to be administered.

The pharmaceutical composition may be administered by any of the known methods, inter alia, per os, intravenous, intraperitoneal, intramuscular or subcutaneous or topical administration.

The present invention further consists in the use of a RY-peptide or of a pharmaceutical composition as defined above in the preparation of a medicament, in particular for the modulation of cell death.

The present invention also consists in a method for the treatment of disorders of inappropriate activation of apoptosis by a RY-peptide or by a pharmaceutical composition as defined above.

The present invention also consists in a method for increasing the number of viable cells in a biological tissue by a RY-peptide or by a pharmaceutical composition as defined above.

A cellular assay system for the activity of the major members of the Bcl-2 family, e.g. Bcl-2 and Bax, and their interactive effects has been constructed in cultured cell lines. (This assay may be used for any member of the Bcl-2 family and for checking the combined effect of each 2 members of this family.) This simple and rapid assay system may be useful in testing the effect of various compounds in modulation of this powerful regulator of cell survival.

This assay is based on transient transfection of cultured cells (e.g., HeLa cells, SH-SY5Y neuroblastoma cells. PC12, pheochromocytoma cells, 293 kidney cells, primary neuronal cells etc.), via electroporation or cationic-lipid- mediated transfection, by an expression vector, harboring a reporter gene (e.g. the gene encoding for beta-galactosidase, or the gene encoding for luciferase). Based on the consideration that gene expression requires high level of function and integrity of cellular systems, a direct correlation can be drawn between the activity of the protein encoded by the reporter gene, and the survival rates of the transfected cells. The reporter gene is co-transfected with a second expression vector, carrying either the bcl-2 or bax genes, therefore affecting the cellular apoptotic threshold towards life or death, respectively. In addition, transfection of cells with a combination of both bcl-2 and bax genes is performed, to examine the activity of each of these two proteins in opposing the death-inhibitory or promoting-effect of the other, respectively.

For the detection of the effects of compounds (hereinafter called "test compounds") as potential modulators of the activity of the Bcl-2 system, each test compound is tested by one of two modes of administration into the cells:

A. Small, membrane permeable test compound particles are administrated by addition to the extracellular medium; and

B. Cell membrane-impermeable small test compound particles are administered by electroporation or by liposome-mediated transfection.

Sould the test compound be a peptide it may be tested by the following mode of administration into the cells:

Small peptides are constructed into expression vectors that

The present invention also consists in a method for enhancement of the survival of biological cells by a RY-peptide or by a pharmaceutical composition as defined above.

Moreover, the present invention further consists in a method for the preparation of a RY-peptide of general formula I which comprises attaching the corresponding amino acids, one after the other, onto a functionalized resin, by the following steps:

a. sythesizing the sequence of Fmoc (9-fluorenyl methoxycarbonyl)-N^{alpha}-protected amino acids activated in situ in a suitable synthesizer and coupling same to a preloaded resin, removing the protecting group and repeating the coupling and deprotecting steps until the entire peptide synthesis has been finalized;

b. cleaving the peptide from the resin,; and

c. purifying the peptide obtained in step b.

The synthesizing step is preferably performed by using an ABI (Applied Biosystems U.K.) 433A synthesizer.

The coupling reagent is preferably HBTU/HOBt (benzotriazole-N,N,N',N'-tetramethyl-uronium hexafuorophosphate / N-hydroxybenzotriazole).

Preferably 3 equivalents of each of the activated amino acid is used in each coupling step

The activation is preferably performed by HBTU/HOBt.

The resin used is a Wang resin or a 2-chlorotrityl resin.

The cleaving operation is preferably performed by acidolysis.

The peptide obtained in step b. is advantageously purified by RP- HPLC (Reversed Phase - High Performance Liquid Chromatography).

The peptide obtained in step c. is advantageously characterized using HPLC, nuclear magnetic resonance (NMR) and/or fast atomic bombardment-mass spectra (FAB-MS).

The sequence of amino acids as defined in general formula 1 may be connected to methionine by methods known per se such as chemical or biological molecular methods.

There has also been developed an in vitro assay system for the evaluation of the regulation of cell death by the Bcl-2 family of proteins. For this purpose:

contain DNA sequences, encoding for the desired peptide; said peptide is transfected into the cells via electroporation or cationic-lipid-mediated transfection.

Potential inhibitors of cell death are evaluated in this system by measuring their ability to overcome bax-induced death process.

Potential triggers of apoptosis are assessed in this system by measuring their ability to induce cell death by themselves, their activity in counteracting Bcl-2 activity, and /or their effect in augmenting Bax cellular toxicity.

The present invention thus also consists in an in vitro assay system for the regulation of cell death by the Bcl-2 family of test compounds (as herein defined), which comprises:

a. transient transfection of cultured cells via electroporation or cationic-lipid mediated transfection by an expression vector, harboring a reporter gene;

b. co-transfecting the reporter gene with a second expression vector, carrying either the death inhibitor or the death inducer genes, thus affecting the cellular apoptotic threshold towards life or death, respectively;

c. performing transfection of cells with a combination of both the death inhibitor and the death inducer genes to examine the activity of each of these two proteins in opposing the death-inhibitory or promoting-effect of the other, respectively;

d. testing the effects of the test compounds as potential modulators of the activity of the Bcl-2 system, by testing each test compound by one of the following two modes of administration into the cells:

e. 1. small, membrane permeable test compound particles are administered by addition to the extracellular medium;

2. cell membrane-impermeable small test compound particles are administered by electroporation or by liposome-mediated transfection;

f. evaluating the potential of the test compounds to inhibit cell death by measuring their ability to

overcome bax-induced death process; and

g. assessing the potential of the test compounds to trigger apoptosis by measuring their ability to induce death by themselves, their activity in counteracting Bcl-2 activity, and /or their effect in augmenting Bax cellular toxicity.

Sould the test compound be a peptide it may be tested by the following mode of administration into the cells:

Small peptides are constructed into expression vectors which contain DNA sequences, encoding for the desired peptide; said peptide being transfected into cells via electroporation or cationic-lipid-mediated transfection;

Said in vitro assay will be exemplified in detail for a test compound being a peptide hereinafter in example 3.b.3.

The present invention will now be illustrated with reference to the following Examples and the accompanying Figs. without being limited by same. (The RY peptide illustrated in the Examples and Figs. is Phe-Glu-Leu-Arg-Tyr-Arg-Arg-Ala-Phe {FELRYRRAF}.)

Examples

Example 1: Synthesis of a RY Peptide:

Peptide synthesis was performed using ABI 433A peptide synthesizer with HBTU/HOBt (benzotriazole-N,N,N',N'-tetramethyl-uronium hexafuorophosphate/N-hydroxybenzotriazole) coupling reagents. Amino acids used were Fmoc (9-fluorenyl methoxycarbonyl)-N^{alpha}-protected. Trifunctional amino acids were side-chain protected as follows: Arg [Pmc (2,2,5,7,8-pentamethyl chroman-6-sulphonyl)], Tyr [tBu (tertbutyl)], Glu [OtBu(terbutyl-ester)]. 330 μmol of preloaded Wang resin were placed in the reaction vessel. Each Fmoc amino acid was activated in situ using HOBt/HBTU, and subsequently coupled to the resin for 50 min. DIEA (Diisopropylethylamine) was used during coupling as a non nucleophilic base. The Fmoc protecting group on the alpha-amine was then removed with 20% piperidine in NMP (N-methyl pyrrolidone) for 20 min. Three equivalents of the activated amino acids were employed in the coupling reactions. The deprotection and

coupling steps were repeated with the addition of each subsequent amino acid until the peptide synthesis was completed. The final amino acid was deprotected using 20% piperidine in NMP, the peptide-resin was washed with NMP, followed by DCM (Dichloromethane), and dried in vacuum.

Cleavage from the solid support

A cleavage mixture consisting of TFA (Trifluoroacetic acid) 97.5% and TIS (Triisopropylsilane) 2.5% was added to the peptide-resin obtained in the previous step (20 ml cleavage mixture to 1 gr resin). The solution was stirred at room temperature for 60 min. The resultant slurry (resin) was filtered using a sintered glass filter. The resin was washed twice with TFA. The filtrate was concentrated to a volume of 1 ml using stream of nitrogen. Following the addition of cold diethyl ether (20 ml) the solution was cooled in an ice bath. After 60 min. the peptide was precipitated by centrifugation, washed with cold ether and dried in vacuum.

Purification and characterization

The peptide obtained in the previous step was purified by RP-HPLC (reversed phase high performance liquid chromatography) on C_{18} 5 μm Phenomenex Kromasil column (10 mm I.D. X 25 cm). Samples were eluted using the following gradient:
(A): distilled H_2O, O.05% TFA; (B): acetonitrile 0.05% TFA; (λ=214 NM; Flow 5 ml/min. The extent of purity for each peptide was monitored by rechromatography on C_{18} 5 μm Phenomenex Kromasil column (4.6 mm I.D. X 25 cm) analytical column, flow 1 ml/min. Characterization of the peptides was performed by FAB-MS (Fast atomic bombardment-Mass spectra).

The Peptide H-Phe-Glu-Leu-Arg-Tyr-Arg-Arg-Ala-Phe-OH:

After purification, the peptide obtained in the previous step was of 86.1% purity, as shown in Figure 2 (non calibrated RP-HPLC, acetonitrile/water 0.1% TFA gradient from 5% to 50% acetonitrile at 30 min). FAB-MS calcd. m/z for $C_{59}H_{89}N_{18}O_{13}$ (MH^+) 1257.7, found 1258.4 (Figure 3).

Example 2: Construction of expression plasmid encoding RY peptide

Construction of RY peptide-coding vector was done by means

of polymerase chain reaction (PCR), and consisted of two steps:

1. **Construction in pIRES-EGFP expression vector.**

The nucleotide sequence encoding the 9 amino acids of the RY peptide was included in a larger oligonucleotide, with both ends matching the sequences of the multi-cloning site of the expression vector pIRES-EGFP (Clontech). The sequence of this oligonucleotide was:

5'-CGACGGAATTCAGTGGATCCATG**TTTGAACTGCGGTACCGGCGCGCGTTC**TAGACTAGTAA-CGGCCGCCAGTG

In this oligonucleotide, the bold and underlined letters designate nucleotides encoding for the RY peptide, and the nucleotides before and after, are homologues to nucleotides 930-949 and 950-969 of the expression vector, respectively. In addition, this oligonucleotide included recognition sites for the restriction enzymes EcoRI (external to the peptide-coding sequence) and PauI (in the peptide coding sequence). In addition, this oligonucleotide also included sequences coding for the amino acid Methionine, immediately preceding the sequence of the RY peptide, to serve as a starting site for protein translation, and a translation stop codon, immediately following the sequence of the RY peptide.

A second oligonucleotide contained sequences homologous to bases 1275-1250 of the expression vector. The sequence of this oligonucleotide was: 5'-GCGTCTAGATGCTCGACCTGCAGTTGGACCTGGG. In this oligonucleotide, the recognition site for the restriction enzyme PstI was introduced, before (5' to) the vector homologous sequences. Both oligonucleotides were used in a PCR reaction, using the vector pIRES-EGFP as a template. PCR reactions were carried out as described by Ho SN et al. (Gene 77:51-59, 1989).

Following the PCR reaction, the reaction product was purified by gel electorphoresis in agarose, followed by purification with a Qiaquick PCR purification kit (Qiagene). The fragment obtained was then digested with the restriction enzymes EcoRI and PstI, and ligated into the vector pIRES-EGFP, digested with the same enzymes. The identity of the RY peptide coding vector was confirmed by DNA sequence analysis.

2. **Construction in the pcDNA3.1 expression vector:**

The DNA fragment encoding for the RY peptide was then

al., Neuropharmacology 35:571-578, 1996). The apoptosis-triggering effect of dopamine has been observed in numerous cell types including Hela cells, and these effects have been shown to be blocked by forced ectopic over expression of Bcl-2 (Ziv I, et al., Apoptosis 2:149-155, 1997; Offen D, et al., Cell Mol Neurobiol 17:289-304. 1997).

2. **Apoptosis induced by hydrogen peroxide**: Exposure to hydrogen peroxide has been shown to induce apoptotic cell death in various cell types (Hoyt KR et al., Neurochem Res 22:333-340, 1997; Li PF et al., 404: 249-252, 1997; Forrest VJ et al., Free Rad Biol Med 16:675-684, 1994).

3. **Apoptosis induced by Bax. This model utilizes an in vitro assay system for regulation of cell death by the Bcl-2 family of proteins**: Cells were grown as described above in example 3a. Cells from log phase of growth were harvested using trypsin / EDTA and resuspended at a density of 5.0 x 10^6 cells/ml in 400 μl PBS. Cell suspension was then transferred to electroporation chambers containing a marker plasmid (pCDNA/Lac z) encoding beta-galactosidase as a reporter gene (10 μg) + a control plasmid (pCDNA), to bring total plasmid quantity to 23 micrograms + one of the following regimens:

1. An expression plasmid, encoding Bcl-2 (0.42 μg).
2. An expression plasmid, encoding Bax (0.42 μg).
3. Bcl-2-encoding plasmid+Bax-encoding plasmid (0.42 μg each).
4. Test compound, or an expression plasmid encoding for the test peptide + either Bcl-2-encoding plasmid or Bax-encoding plasmid or both.
5. Control: only the marker plasmid.

After one minute of incubation, electroporation was performed at 1050 μF and 220 V for a duration of 10-30 ms, using an Equibio gene pulser. Cells from the cuvettes were then pooled into a complete medium with 10% FCS, and 1x10^6 cells were aliquoted into 3.5 cm plates. At 18 hours post-transfection, cell viability was assessed through measurement of beta-galactosidase activity by standard methods (Sambrook, Fritch, Maniatis, Molecular cloning; A laboratory

subcloned into the expression vector pcDNA3.1, following its rescue from pIRES-EGFP by digestion with EcoRI and MscI restriction enzymes. The fragment was purified as described in the above step 1, and ligated into pcDNA3.1 that was precut with HindIII, filled-in with Klenow enzyme, and then digested with EcoRI. Identity of the RY peptide-encoding plasmid was verified by restriction with PauI, an enzyme having a unique restriction site present only in the RY-peptide coding sequence. This vector was used for the transfection studies. All standard molecular biology procedures were carried out as described in Sambrook et al. (Sambrook, Fritch, Maniatis, Molecular cloning; A laboratory manual, Cold Spring Harbour Laboratory Press, pp 16.66, 1989).

Example 3: **Methods for evaluation of the cell survival-promoting effect of RY peptide**

a. **Cell cultures:**

1. HeLa cells: Human cervical carcinoma cells (HeLa) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin-Nystatin solution (Biological Industries, Israel), and 1% L-Glutamine solution (2 mM, biological Industries, Israel). Cells were grown in monolayer at 37°C in 5% CO_2.

2. Kidney 293 cells (T): Human embryonic kidney 293 cells were grown in the conditions described for the HeLa cells.

b. **Models of apoptosis:**

Three model systems of cell death were used:

1. **Apoptosis induced by dopamine:** Exposure of cells to dopamine at 100-500 micromolar for 24 hours induces cell death characteristic of apoptosis (Ziv I, et al., Neurosci Lett 170:136-140, 1994; Offen D, et al., Biochim Biophys Acta 1268:171-177, 1995; Masserano JM, et al., Mol Pharmacol 50:1309-1315, 1996; Shirvan A, et al., J. Neurochem 69:539-549, 1997; Corona V., et al., J. Neurochem 69:1870-1881, 1997; Velez-Pardo C, et al., Pharmacol Toxicol 80:76-84, 1997; Shinkai T, et al., J Neurosci Res. 393-399, 1997; Simantov R, et al., Neuroscience 74:39-50, 1996; Gabby M, et

manual, Cold Spring Harbour Laboratory Press, pp 16.66, 1989). OD values were expressed as percent of control, and statistical significance was evaluated by appropriate parametric or non-parametric statistical tests.

As shown in Figure 4, induced-expression of Bcl-2 in this system increases cell viability. Conceivably, it reflects the activity of Bcl-2 in inhibition of naturally-occurring cell death in culture and/or inhibition of cell death induced by the electroporation procedure. By contrast, as also shown in Figure 4, Bax manifests a marked death-inducing effect, and this cell-killing effect of Bax can be markedly counteracted by co-transfection with Bcl-2. This in vitro assay system therefore reproduces effectively the expected activity of these major members of the Bcl-2 family (Oltvai ZN et al., Cell 74:609-619, 1993; Han J et al., Genes Dev 10:461-477, 1996; Zha H et al., Mol Cell Biol 16:6494-6508, 1996).

Example 4: RY peptide inhibits dopamine-induced cell death

The objective of the experiment was to evaluate the ability of RY peptide to inhibit cell death induced by dopamine in HeLa cells.

Methods: RY peptide was synthesized as described above in Example 1, and introduced into the cells with the reporter plasmid pCDNA/Lac-z, by the electroporation procedure as described above in Example 3.b.2. Five hours after electroporation, cells were exposed to dopamine (150μM and 300μM, Sigma, Ltd., Israel) for 18 hours. Cell viability was then evaluated by the measurement of the beta-galactosidase activity, as specified above.

Results: As described in Figure 5, dopamine induced a dose-dependent cell death, reflected by reduction of cell survival to 30.8 + 6.0 and 8.4 + 7.0 percent of control, following exposure to 150 and 300 μM of dopamine, respectively, for 18 hours. RY peptide manifested a marked protective effect against dopamine toxicity, raising cell survival rates to 75.4+9.0 % and 40.2+ 8.0% respectively ($P<0.001$).

Conclusion: RY peptide is a potent inhibitor of dopamine-induced cell death, as evaluated in this experimental system.

Example 5: RY peptide increases cell survival rate and inhibits Bax-induced cell death, thus mimicking the effect of Bcl-2.

The objective of this experiment was to test the effect of RY peptide on cell survival, in the in vitro assay for regulation of cell death by the Bcl-2 system.

Methods: An expression plasmid, encoding the RY peptide (synthesized as described above in Example 2) was used. The plasmid was then introduced into the assay system of the Bcl-2 proteins, as described above in Example 3.b.3.

Results: As shown in Figure 6, cells transfected by electroporation with RY peptide manifested markedly enhanced cell viability as compared to controls, similar to the effect of Bcl-2 (Figure 4). Moreover, RY peptide tranfection conferred marked protection against Bax death-inducing effect, reflected by a cell survival rate of 71.4 + 9.4% of control, versus 37.0 + 2.2% ($p<0.001$), similar to the effect of Bcl-2.

Conclusion: As evaluated in this experimental system, RY increases cell survival following electroporation and inhibits the death-inducing effect of Bax. These effects may be therefore regarded as mimicking the effect of Bcl-2.

Example 6: RY peptide inhibits cell-killing effect of Bax on embryonic kidney 293T cells

The objective of this experiment was to show the effect of RY peptide in counteracting the cell-killing effect of Bax, in a different model system (non-HeLa cells, non-electroporation-mediated transfection).

Methods: Human embryonic kidney 293T cells were grown as specified in Example 3.a. Cells were co-transfected using the Superfect Transfection Kit (Quiagen), with 5 μg total DNA of the following effectors: pCDNA/Lac-z reporter plasmid and one of the following:

1). Expression plasmid encoding RY peptide.

2). Expression plasmid encoding Bax.

3). Expression plasmids, encoding Bax and RY peptide.

After 18 hours, the cells were harvested for beta-galactosidase activity assay.

Results: As shown in Figure 7, the RY peptide was markedly active in counteracting Bax toxicity.

Conclusion: RY peptide is a potent inhibitor of Bax cell-killing effect in this experimental system.

Example 7: RY peptide inhibits hyfrogen peroxide toxicity in Hela cells

The objective of this experiment was to examine the ability of RY peptide to inhibit cell death induced by hydrogen peroxide in HeLa cells.

Methods: Two methods for administration of RY peptide were employed:

1) Hela cells were co-transfected (via electroporation), with two expression vectors: (1) Expression vector encoding for the reporter gene beta-galactosidase. (2). Expression vector encoding for RY peptide (as described in Example 2).

2) Hela cells were co-transfected (via electroporation), with the expression vector encoding for beta-galactosidase and with the synthetic RY peptide (as described in Example 1).

Five hours after the electroporation procedure, cells were tested with 100μM and 200μM of hydrogen peroxide (Merck, Inc. USA), for 18 hours.

As described in Figures 8A and 8B, hydrogen peroxide treatment reduced cell survival rate to 20.4±2.8 and 5.8±2.1 percent of control (mean ± SD), respectively. RY peptide, both plasmid-encoded and synthetic peptide, manifested a marked protective effect. Cell survival rate in the presence of hydrogen peroxide and plasmid-encoded RY peptide was increased to 53.9±9.3 and 15.3±4.2 percent of control, respectively. Protective effect of synthetic RY peptide was reflected by increase of cell survival rate to 53.3±8.8 and 33.4±3.7%, respectively ($p<0.001$).

Figure Legends:

Figure 1: Structure of the RY region

NMR solution three-dimensional structure of Bcl-xL (Muchmore SW, et al., Nature 381: 335-341, 1996) was extracted from the Brookhaven Protein Data Bank, Accession No. 1LXL. The RY region (Phe97-Ph105), is marked. As shown, this small region is located on the outer surface of the protein, forming an almost closed loop.

Figure 2: RP-HPCL of the purified RY peptide.

RP-HPLC (Reversed phase high performance liquid chromatography) of purified RY peptide (H-Phe-Glu-Leu-Arg- Tyr-Arg- Arg-Ala-Phe-OH). The peptide was analysed on C_{18}-5 μm Phenomenex Kromasil column (4.6 mm. I.D. X 25 cm). A sample was eluted using the following gradient: A, distilled H_2O 0.05% TFA (Trifluoroacetic acid)/;Acetonitrile 0.05% TFA; λ=214; flow 1 ml/min. t=0%B=5 t=30min %B=50. The numbers on the y axis indicate mili absorbance units. The numbers on the X axis indicate minutes. The numbers on the peaks of the graph indicate the retention time.

Figure 3: MS-(FAB) of the purified RY peptide

MS-(FAB) (Mass spectra-Fast atomic bombardment) of purified H-Phe-Glu-Leu-Arg-Tyr-Arg-Arg-Ala-Phe-OH. The numbers on the y axis indicate the relative ion abundance of the ms detector. On the x axis the mass per charge M/z is calculated for $C_{59}H_{89}N_{18}O_{13}$ (MH^+) 1257.7, and the M/z found was 1258.4.

Figure 4: In vitro assay system for the Bcl-2 family of proteins

HeLa cells were co-transfected with a beta-galactosidase reporter plasmid in combination with either a control plasmid (vector), a plasmid expressing Bcl-2 or Bax, or both plasmids expressing Bcl-2 and Bax, using electroporation procedure. Cell viability was assessed at 18 hours past the electroperotion, by measurement of beta-galactosidase activity.

Figure 5: RY peptide protects HeLa cells against dopamine toxicity:

Synthetic RY peptide was introduced into HeLa cells with a

beta-galactosidase reporter plasmid by electroporation. After five hours, cells were exposed to dopamine (150μu and 300μM, Sigma, Ltd., Israel) for 18 hours. Cell viability was then evaluated by measurement of the beta-galactosidase activity.

Figure 6: RY peptide increases cell survival rate and inhibits Bax-induced cell death, thus mimicking the effect of Bcl-2.

HeLa cells were co-transfected by electroporation with an expression plasmid encoding the RY peptide and a beta-galactosidase reporter plasmid, and introduced into the assessment system of the Bcl-2 proteins, as described above in Example 3.b.3 and Figure 4. Cell viability was evaluated 18 hours after the electroporation by measurement of beta-galactosidase activity.

Figure 7: RY peptide inhibits cell-killing effect of Bax on embryonic kidney 293T cells

Human embryonic kidney 293T cells were co-transfected by electroporation with a reporter plasmid encoding beta-galactosidase, in combination with either an expression plasmid encoding RY peptide; an expression plasmid encoding Bax; or expression plasmids encoding Bax and RY peptide. Cell viability was evaluated after 18 hours, by measurement of beta-galactosidase activity.

Figure 8A: Plasmid-encoded RY peptide protects against hydrogen peroxide toxicity.

HeLa cells were co-transfected via electroporation with an expression plasmid encoding for RY peptide, together with a reporter plasmid encoding for beta-galactosidase. Five hours after electroporation, cells were treated with either 100μM or 200μM of hydrogen peroxide (HP) for 18 hours. Cell viability was then evaluated by determining beta-galactosidase activity.

Figure 8B: Synthetic RY peptide protects against hydrogen peroxide toxicity.

HeLa cells were co-transfected via electroporation with RY

synthetic peptide, together with a reporter plasmid encoding for beta-galactosidase. Five hours after electroporation, cells were treated with either 100μM or 200μM of hydrogen peroxide (HP) for 18 hours. Cell viability was then evaluated by determining beta-galactosidase activity.

Claims:

1. An isolated and purified peptide of the RY domain having an amino acid sequence of general formula I comprising a sequence of the following amino acids:

$$X_1 - X_2 - X_3 - X_4 - X_1 - X_4 - X_4 - X_3 - X_1 \qquad I$$

X_1 = Phe, Tyr, or any amino acid having a substituted aromatic residue;

X_2 = Glu, Asp, Ser, or any amino acid having a $-(CH_2)_n-COO^-$ residue, wherein n = 0-3;

X_3 = Asp, Thr, any aliphatic amino acid, or any of amino acids X_4; and

X_4 = Arg, Lys, or any amino acid having a $-(CH_2)_n-NH_3^+$ residue, or a $-(CH_2)_n-NH-C(NH_3^+)NH_2$ residue wherein n = 0-4;

as well as functional equivalents thereof.

2. A peptide according to Claim 1, wherein Methionine (Met) is connected to the N-terminal of the sequence of general formula I.
3. A peptide according to Claim 1 or 2 wherein in the sequence of general formula I, the sequence is
$X_4 - X_1 - X_4 - X_4$ stands for Arg - Tyr - Arg - Arg.
4. A peptide according to Claim 3, wherein the sequence is preceded by X_3 = Arg.
5. A peptide according to any of Claims 1 to 4, wherein the substituted aromatic residue of X_1 is Phenyl-$(CH_2)_n$-.
6. A peptide according to any of Claims 1 to 5, wherein the aliphatic amino acid of X_3 is selected among Leu, Ile, Ala, Gly and Val.
7. Phe-Glu-Leu-Arg-Tyr-Arg-Arg-Ala-Phe.
8. Phe-Ser-Arg-Arg-Tyr-Arg-Arg-Asp-Phe.
9. Phe-Glu-Thr-Arg-Phe-Arg-Arg-Thr-Phe.
10. A pharmaceutical composition comprising as active ingredient a RY peptide according to any of Claims 1 to 9 or a functional equivalent thereof.
11. A pharmaceutical composition according to Claim 10 comprising a pharmaceutical acceptable carrier.
12. The use of the RY peptide or of a pharmaceutical composition according to any of Claims 1 to 11 in the preparation of a

medicament.

13. A method for the treatment of disorders of inappropriate activation of apotosis by a RY peptide or by a pharmaceutical composition according to any of Claims 1 to 11.

14. A method for increasing the number of viable cells in a biological tissue by a RY-peptide or by a pharmaceutical composition according to any of Claims 1 to 11.

15. A method for the enhancement for the survival of biological cells by a RY-peptide or by a pharmaceutical composition according to any of Claims 1 to 11.

16. A method for the preparation of a RY-peptide of general formula I according to Claim 1, which comprises attaching the corresponding amino acids, one after the other, onto a functionalized resin, by the following steps:

a. sythesising the sequence of Fmoc (9-fluorenyl methoxycarbonyl)-N^{alpha}-protected amino acids activated in situ in a suitable synthesizer and coupling same to a preloaded resin, removing the protecting group and repeating the coupling and deprotecting steps until the entire peptide synthesis has been finalized;

b. cleaving the peptide from the resin,; and

c. purifying the peptide obtained in step b.

17. A method according to Claim 16, wherein the synthesizing step is performed by using an ABI (Applied Biosystems U.K.) 433 A synthesizer.

18. A method according to Claim 16 or 17, wherein the coupling reagent is HBTU/HOBt (benzotriazole-N,N,N',N'-tetramethyl-uronium hexafuorophosphate/N-hydroxybenzotriazole).

19. A method according to any of Claims 16 to 18, wherein 3 equivalents of each of the activated amino acids is used in each coupling step.

20. A method according to any of Claims 16 to 19, wherein the resin is selected among a Wang resin and a 2-chlorotrityl resin.

21. An isolated and purified peptide of the RY domain having an amino acid sequence of general formula I as defined in Claim 1 and 2, substantially as herein described with reference to the examples.

22. An in vitro assay system for the regulation of cell death by the Bcl-2 family of test compounds (as hereinbefore defined), which comprises:

a. transient transfection of cultured cells via electroporation or cationic-lipid mediated transfection by an expression vector, harboring a reporter gene;

b. co-transfecting the reporter gene with a second expression vector, carrying either the death inhibitor or the death inducer genes, thus affecting the cellular apoptotic threshold towards life or death, respectively;

c. performing transfection of cells with a combination of both the death inhibitor and the death inducer genes to examine the activity of each of these two proteins in opposing the death-inhibitory or promoting-effect of the other, respectively;

d. testing the effects of the test compounds as potential modulators of the activity of the Bcl-2 system, by testing each test compound by one of the following two modes of administration into the cells:

e. 1. small, membrane permeable test compound particles are administered by addition to the extracellular medium;

2. cell membrane-impermeable small test compound particles are administered by electroporation or by liposome-mediated transfection;

f. evaluating the potential of the test compounds to inhibit cell death by measuring their ability to overcome bax-induced death process; and

g. assessing the potential of the test compounds to trigger apoptosis by measuring their ability to induce death by themselves, their activity in counteracting Bcl-2 activity, and /or their effect in augmenting Bax cellular toxicity.

23. An in vitro assay according to Claim 22, wherein the test compound is a peptide.

24. An in vitro assay according to Claim 23, wherein the peptide may be tested in addition by constructing small peptides

into expression vectors which contain DNA sequences, encoding for the desired peptide; said peptide being transfected into cells via electroporation or cationic-lipid-mediated transfection.

25. An in vitro assay system as defined in Claim 22, substantially as described in Example 3.b.3.

22. An in vitro assay system for the regulation of cell death by the Bcl-2 family of test compounds (as hereinbefore defined), which comprises:
 a. transient transfection of cultured cells via electroporation or cationic-lipid mediated transfection by an expression vector, harboring a reporter gene;
 b. co-transfecting the reporter gene with a second expression vector, carrying either the death inhibitor or the death inducer genes, thus affecting the cellular apoptotic threshold towards life or death, respectively;
 c. performing transfection of cells with a combination of both the death inhibitor and the death inducer genes to examine the activity of each of these two proteins in opposing the death-inhibitory or promoting-effect of the other, respectively;
 d. testing the effects of the test compounds as potential modulators of the activity of the Bcl-2 system, by testing each test compound by one of the following two modes of administration into the cells:
 e. 1. small, membrane permeable test compound particles are administered by addition to the extracellular medium;
 2. cell membrane-impermeable small test compound particles are administered by electroporation or by liposome-mediated transfection;
 f. evaluating the potential of the test compounds to inhibit cell death by measuring their ability to overcome bax-induced death process; and
 g. assessing the potential of the test compounds to trigger apoptosis by measuring their ability to induce death by themselves, their activity in counteracting Bcl-2 activity, and /or their effect in augmenting Bax cellular toxicity.

23. An in vitro assay according to Claim 22, wherein the test compound is a peptide.

24. An in vitro assay according to Claim 23, wherein the peptide may be tested in addition by constructing small peptides

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into expression vectors which contain DNA sequences, encoding for the desired peptide; said peptide being transfected into cells via electroporation or cationic-lipid-mediated transfection.

25. An in vitro assay system as defined in Claim 22, substantially as described in Example 3.b.3.

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Brookhaven Protein Data Bank, Accession No. 1LXL and 1MAZ, respectively). These structural data also reveal that this small region forms an almost closed loop within the protein structure, with the Phe1 and Phe9 in close spatial proximity, while the Arg side-chains face the outside of the protein (Figure 1).

4. Undergoing conformational change upon binding of Bcl-xL to a peptide from the BH3 region of a death inducer (Bak): A systematic comparison has been performed of the structure of the free Bcl-xL (Muchmore SW, et al., Nature 381:335-341, 1996) and its structure upon binding to the BH3 peptide of Bak (Sattler M, et al., Science 275:983-986, 1997). In its unbound conformation, the second helix of the protein encompasses 15 amino acids (Ala84-Glu98). The RY domain in this conformation is the loop described above (Fig. 1.) However, upon binding of the BH3 peptide of Bak to the hydrophobic cleft of Bcl-xL, the RY domain undergoes a conformational change and becomes a part of the second helix, which now extends to residue Arg103 (Sattler M, et al., Science 275:983-986, 1997). Moreover, upon binding, several of the RY residues become engaged in interactions with the death-inducer peptide (i.e., Phe97,Arg100, Tyr101, Phe105).

5. Markedly different in cell death inhibitors vs. inducers:
 Death inhibitors:
 Bcl-xL: FELRYRRAF (Phe-Glu-Leu-Arg-Tyr-Arg-Arg-Ala-Phe)
 Bcl-2: FSRRYRRDF (Phe-Ser-Arg-Arg-Tyr-Arg-Arg-Asp-Phe)
 Bcl-w: FETRFRRTF (Phe-Glu-Thr-Arg-Phe-Arg-Arg-Thr-Phe)
 Death inducers:
 Bax: LDSNMEL (Leu-Asp-Ser-Asn-Met-Glu-Leu)
 Bak: INRRYDSEF (Ile-Asn-Arg-Arg-Tyr-Asp-Ser-Glu-Phe)
 It has been found that the peptide FELRYRRAF is a potent inhibitor of cell death.

The present invention thus consists in an isolated and purified peptide of the RY domain having an amino acid sequence of general formula I comprising a sequence of the following amino acids:

$$X_1 - X_2 - X_3 - X_4 - X_1 - X_4 - X_4 - X_3 - X_1 \qquad \text{I}$$

X_1 = Phe, Tyr, or any amino acid having a substituted

aromatic residue;

X_2 = Glu, Asp, Ser, or any amino acid having a $-(CH_2)_n-COO^-$ residue, wherein n = 0-3;

X_3 = Asp, Thr, any aliphatic amino acid, or any of amino acids X_4; and

X_4 = Arg, Lys, or any amino acid having a $-(CH_2)_n-NH_3^+$ residue, or a $-(CH_2)_n-NH-C(NH_3^+)NH_2$ residue wherein n = 0-4;

as well as functional equivalents thereof.

By "functional equivalents" is meant a compound possessing a biological activity or/and immunological characteristic similar to that of the RY domain of general formula I. Said term includes fragments, variants analogs, homologs and chemical derivatives possessing such activity or characteristic.

Any of the above amino acids may be either the D- or the L-isomer.

The amino acid residues may also be residues of suitable synthetic amino acids.

Methionine (Met) may be connected to the N-terminal of the sequence of general formula I and this sequence is also within the scope of the present invention.

In an advantageous sequence of general formula I the sequence

X_4 - X_1 - X_4 - X_4 stands for Arg - Tyr - Arg - Arg;

In a further embodiment said sequence is preceded by X_3 = Arg.

The substituted aromatic residue of X_1 is preferably Phenyl-$(CH_2)_n$-, wherein n = 0-3.

The aliphatic amino acid of X_3 is preferably selected among Leu, Ile, Ala, Gly and Val.

The preferred sequence according to the present invention is Phe-Glu-Leu-Arg-Tyr-Arg-Arg-Ala-Phe (FELRYRRAF).

Said sequence corresponds to residues 97 - 105 of the Bcl-xL protein.

Additional sequences which may be considered as suitable death inhibitors are:

Phe-Ser-Arg-Arg-Tyr-Arg-Arg-Asp-Phe (FSRRYRRDF)

Said sequence corresponds to residues 104 - 112 of the Bcl-2

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protein.

Phe-Glu-Thr-Arg-Phe-Arg-Arg-Thr-Phe (FETRFRRTF).

Said sequence corresponds to residues 53 - 61 of the Bcl-w protein.

The present invention consists also in pharmaceutical compositions comprising as active ingredient of the RY- peptide, as defined above.

In a preferred embodiment the pharmaceutical composition comprises in addition to the RY-peptide a pharmaceutically acceptable carrier.

The pharmaceutical compositions may be, e.g. tablets, capsules, solutions, emulsions, etc. The carriers may be selected among any suitable components, e.g. solvents; emulgators; excipients; talc; flavors; colors; etc. The pharmaceutical composition may comprise, if desired, also other pharmaceutically active compounds.

The amount of the RY protein incorporated in the pharmaceutical composition may vary widely. The factors which have to be considered when determining the precise amount are known to those skilled in the art. Such factors include, inter alia, the pharmaceutical carrier being part of the composition, the route of administration being employed and the frequency with which the composition is to be administered.

The pharmaceutical composition may be administered by any of the known methods, inter alia, per os, intravenous, intrapertioneal, intramuscular or subcutaneous or topical administration.

The present invention further consists in the use of a RY-peptide or of a pharmaceutical composition as defined above in the preparation of a medicament, in particular for the modulation of cell death.

The present invention also consists in a method for the treatment of disorders of inappropriate activation of apoptosis by a RY-peptide or by a pharmaceutical composition as defined above.

The present invention also consists in a method for increasing the number of viable cells in a biological tissue by a RY-peptide or by a pharmaceutical composition as defined above.

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The present invention also consists in a method for enhancement of the survival of biological cells by a RY-peptide or by a pharmaceutical composition as defined above.

Moreover, the present invention further consists in a method for the preparation of a RY-peptide of general formula I which comprises attaching the corresponding amino acids, one after the other, onto a functionalized resin, by the following steps:

a. sythesizing the sequence of Fmoc (9-fluorenyl methoxycarbonyl)-N^{alpha}-protected amino acids activated in situ in a suitable synthesizer and coupling same to a preloaded resin, removing the protecting group and repeating the coupling and deprotecting steps until the entire peptide synthesis has been finalized;

b. cleaving the peptide from the resin,; and

c. purifying the peptide obtained in step b.

The synthesizing step is preferably performed by using an ABI (Applied Biosystems U.K.) 433A synthesizer.

The coupling reagent is preferably HBTU/HOBt (benzotriazole-N,N,N',N'-tetramethyl-uronium hexafuorophosphate / N-hydroxybenzotriazole).

Preferably 3 equivalents of each of the activated amino acid is used in each coupling step

The activation is preferably performed by HBTU/HOBt.

The resin used is a Wang resin or a 2-chlorotrityl resin.

The cleaving operation is preferably performed by acidolysis.

The peptide obtained in step.b. is advantageously purified by RP- HPLC (Reversed Phase - High Performance Liquid Chromatography).

The peptide obtained in step c. is advantageously characterized using HPLC, nuclear magnetic resonance (NMR) and/or fast atomic bombardment-mass spectra (FAB-MS).

The sequence of amino acids as defined in general formula 1 may be connected to methionine by methods known per se such as chemical or biological molecular methods.

There has also been developed an in vitro assay system for the evaluation of the regulation of cell death by the Bcl-2 family of proteins. For this purpose:

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overcome bax-induced death process; and

g. assessing the potential of the test compounds to trigger apoptosis by measuring their ability to induce death by themselves, their activity in counteracting Bcl-2 activity, and /or their effect in augmenting Bax cellular toxicity.

Sould the test compound be a peptide it may be tested by the following mode of administration into the cells:

Small peptides are constructed into expression vectors which contain DNA sequences, encoding for the desired peptide; said peptide being transfected into cells via electroporation or cationic-lipid-mediated transfection;

Said in vitro assay will be exemplified in detail for a test compound being a peptide hereinafter in example 3.b.3.

The present invention will now be illustrated with reference to the following Examples and the accompanying Figs. without being limited by same. (The RY peptide illustrated in the Examples and Figs. is Phe-Glu-Leu-Arg-Tyr-Arg-Arg-Ala-Phe {FELRYRRAF}.)

Examples

Example 1: Synthesis of a RY Peptide:

Peptide synthesis was performed using ABI 433A peptide synthesizer with HBTU/HOBt (benzotriazole-N,N,N',N'-tetramethyl-uronium hexafuorophosphate/N-hydroxybenzotriazole) coupling reagents. Amino acids used were Fmoc (9-fluorenyl methoxycarbonyl)-N^{alpha}-protected. Trifunctional amino acids were side-chain protected as follows: Arg [Pmc (2,2,5,7,8-pentamethyl chroman-6-sulphonyl)], Tyr [tBu (tertbutyl)], Glu [OtBu(terbutyl-ester)]. 330 μmol of preloaded Wang resin were placed in the reaction vessel. Each Fmoc amino acid was activated in situ using HOBt/HBTU, and subsequently coupled to the resin for 50 min. DIEA (Diisopropylethylamine) was used during coupling as a non nucleophilic base. The Fmoc protecting group on the alpha-amine was then removed with 20% piperidine in NMP (N-methyl pyrrolidone) for 20 min. Three equivalents of the activated amino acids were employed in the coupling reactions. The deprotection and

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coupling steps were repeated with the addition of each subsequent amino acid until the peptide synthesis was completed. The final amino acid was deprotected using 20% piperidine in NMP, the peptide-resin was washed with NMP, followed by DCM (Dichloromethane), and dried in vacuum.

Cleavage from the solid support

A cleavage mixture consisting of TFA (Trifluoroacetic acid) 97.5% and TIS (Triisopropylsilane) 2.5% was added to the peptide-resin obtained in the previous step (20 ml cleavage mixture to 1 gr resin). The solution was stirred at room temperature for 60 min. The resultant slurry (resin) was filtered using a sintered glass filter. The resin was washed twice with TFA. The filtrate was concentrated to a volume of 1 ml using stream of nitrogen. Following the addition of cold diethyl ether (20 ml) the solution was cooled in an ice bath. After 60 min. the peptide was precipitated by centrifugation, washed with cold ether and dried in vacuum.

Purification and characterization

The peptide obtained in the previous step was purified by RP-HPLC (reversed phase high performance liquid chromatography) on C_{18} 5 μm Phenomenex Kromasil column (10 mm I.D. X 25 cm). Samples were eluted using the following gradient:
(A): distilled H_2O, 0.05% TFA; (B): acetonitrile 0.05% TFA; (λ=214 NM; Flow 5 ml/min. The extent of purity for each peptide was monitored by rechromatography on C_{18} 5 μm Phenomenex Kromasil column (4.6 mm I.D. X 25 cm) analytical column, flow 1 ml/min. Characterization of the peptides was performed by FAB-MS (Fast atomic bombardment-Mass spectra).

The Peptide H-Phe-Glu-Leu-Arg-Tyr-Arg-Arg-Ala-Phe-OH:

After purification, the peptide obtained in the previous step was of 86.1% purity, as shown in Figure 2 (non calibrated RP-HPLC, acetonitrile/water 0.1% TFA gradient from 5% to 50% acetonitrile at 30 min). FAB-MS calcd. m/z for $C_{59}H_{89}N_{18}O_{13}$ (MH^+) 1257.7, found 1258.4 (Figure 3).

Example 2: Construction of expression plasmid encoding RY peptide

Construction of RY peptide-coding vector was done by means

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of polymerase chain reaction (PCR), and consisted of two steps:

1. Construction in pIRES-EGFP expression vector.

The nucleotide sequence encoding the 9 amino acids of the RY peptide was included in a larger oligonucleotide, with both ends matching the sequences of the multi-cloning site of the expression vector pIRES-EGFP (Clontech). The sequence of this oligonucleotide was:

5'-CGACGGAATTCAGTGGATCCATG**TTTGAACTGCGGTACCGGCGCGCGTTC**TAGACTAGTAA-CGGCCGCCAGTG

In this oligonucleotide, the bold and underlined letters designate nucleotides encoding for the RY peptide, and the nucleotides before and after, are homologues to nucleotides 930-949 and 950-969 of the expression vector, respectively. In addition, this oligonucleotide included recognition sites for the restriction enzymes EcoRI (external to the peptide-coding sequence) and PauI (in the peptide coding sequence). In addition, this oligonucleotide also included sequences coding for the amino acid Methionine, immediately preceding the sequence of the RY peptide, to serve as a starting site for protein translation, and a translation stop codon, immediately following the sequence of the RY peptide.

A second oligonucleotide contained sequences homologous to bases 1275-1250 of the expression vector. The sequence of this oligonucleotide was: 5'-GCGTCTAGATGCTCGACCTGCAGTTGGACCTGGG. In this oligonucleotide, the recognition site for the restriction enzyme PstI was introduced, before (5' to) the vector homologous sequences. Both oligonucleotides were used in a PCR reaction, using the vector pIRES-EGFP as a template. PCR reactions were carried out as described by Ho SN et al. (Gene 77:51-59, 1989).

Following the PCR reaction, the reaction product was purified by gel electorphoresis in agarose, followed by purification with a Qiaquick PCR purification kit (Qiagene). The fragment obtained was then digested with the restriction enzymes EcoRI and PstI, and ligated into the vector pIRES-EGFP, digested with the same enzymes. The identity of the RY peptide coding vector was confirmed by DNA sequence analysis.

2. Construction in the pcDNA3.1 expression vector:

The DNA fragment encoding for the RY peptide was then

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subcloned into the expression vector pcDNA3.1, following its rescue from pIRES-EGFP by digestion with EcoRI and MscI restriction enzymes. The fragment was purified as described in the above step 1, and ligated into pcDNA3.1 that was precut with HindIII, filled-in with Klenow enzyme, and then digested with EcoRI. Identity of the RY peptide-encoding plasmid was verified by restriction with PauI, an enzyme having a unique restriction site present only in the RY-peptide coding sequence. This vector was used for the transfection studies. All standard molecular biology procedures were carried out as described in Sambrook et al. (Sambrook, Fritch, Maniatis, Molecular cloning; A laboratory manual, Cold Spring Harbour Laboratory Press, pp 16.66, 1989).

Example 3: Methods for evaluation of the cell survival-promoting effect of RY peptide

a. **Cell cultures:**

1. HeLa cells: Human cervical carcinoma cells (HeLa) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin-Nystatin solution (Biological Industries, Israel), and 1% L-Glutamine solution (2 mM, biological Industries, Israel). Cells were grown in monolayer at 37°C in 5% CO_2.

2. Kidney 293 cells (T): Human embryonic kidney 293 cells were grown in the conditions described for the HeLa cells.

b. **Models of apoptosis:**

Three model systems of cell death were used:

1. **Apoptosis induced by dopamine:** Exposure of cells to dopamine at 100-500 micromolar for 24 hours induces cell death characteristic of apoptosis (Ziv I, et al., Neurosci Lett 170:136-140, 1994; Offen D, et al., Biochim Biophys Acta 1268:171-177, 1995; Masserano JM, et al., Mol Pharmacol 50:1309-1315, 1996; Shirvan A, et al., J. Neurochem 69:539-549, 1997; Corona V., et al., J. Neurochem 69:1870-1881, 1997; Velez-Pardo C, et al., Pharmacol Toxicol 80:76-84, 1997; Shinkai T, et al., J Neurosci Res. 393-399, 1997; Simantov R, et al., Neuroscience 74:39-50, 1996; Gabby M, et

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manual, Cold Spring Harbour Laboratory Press, pp 16.66, 1989). OD values were expressed as percent of control, and statistical significance was evaluated by appropriate parametric or non-parametric statistical tests.

As shown in Figure 4, induced-expression of Bcl-2 in this system increases cell viability. Conceivably, it reflects the activity of Bcl-2 in inhibition of naturally-occurring cell death in culture and/or inhibition of cell death induced by the electroporation procedure. By contrast, as also shown in Figure 4, Bax manifests a marked death-inducing effect, and this cell-killing effect of Bax can be markedly counteracted by co-transfection with Bcl-2. This in vitro assay system therefore reproduces effectively the expected activity of these major members of the Bcl-2 family (Oltvai ZN et al., Cell 74:609-619, 1993; Han J et al., Genes Dev 10:461-477, 1996; Zha H et al., Mol Cell Biol 16:6494-6508, 1996).

Example 4: RY peptide inhibits dopamine-induced cell death

The objective of the experiment was to evaluate the ability of RY peptide to inhibit cell death induced by dopamine in HeLa cells.

Methods: RY peptide was synthesized as described above in Example 1, and introduced into the cells with the reporter plasmid pCDNA/Lac-z, by the electroporation procedure as described above in Example 3.b.2. Five hours after electroporation, cells were exposed to dopamine (150μM and 300μM, Sigma, Ltd., Israel) for 18 hours. Cell viability was then evaluated by the measurement of the beta-galactosidase activity, as specified above.

Results: As described in Figure 5, dopamine induced a dose-dependent cell death, reflected by reduction of cell survival to 30.8 + 6.0 and 8.4 + 7.0 percent of control, following exposure to 150 and 300 μM of dopamine, respectively, for 18 hours. RY peptide manifested a marked protective effect against dopamine toxicity, raising cell survival rates to 75.4+9.0 % and 40.2+ 8.0% respectively ($P<0.001$).

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Conclusion: RY peptide is a potent inhibitor of dopamine-induced cell death, as evaluated in this experimental system.

Example 5: RY peptide increases cell survival rate and inhibits Bax-induced cell death, thus mimicking the effect of Bcl-2.

The objective of this experiment was to test the effect of RY peptide on cell survival, in the in vitro assay for regulation of cell death by the Bcl-2 system.

Methods: An expression plasmid, encoding the RY peptide (synthesized as described above in Example 2) was used. The plasmid was then introduced into the assay system of the Bcl-2 proteins, as described above in Example 3.b.3.

Results: As shown in Figure 6, cells transfected by electroporation with RY peptide manifested markedly enhanced cell viability as compared to controls, similar to the effect of Bcl-2 (Figure 4). Moreover, RY peptide tranfection conferred marked protection against Bax death-inducing effect, reflected by a cell survival rate of 71.4 + 9.4% of control, versus 37.0 + 2.2% ($p<0.001$), similar to the effect of Bcl-2.

Conclusion: As evaluated in this experimental system, RY increases cell survival following electroporation and inhibits the death-inducing effect of Bax. These effects may be therefore regarded as mimicking the effect of Bcl-2.

Example 6: RY peptide inhibits cell-killing effect of Bax on embryonic kidney 293T cells

The objective of this experiment was to show the effect of RY peptide in counteracting the cell-killing effect of Bax, in a different model system (non-HeLa cells, non-electroporation-mediated transfection).

Methods: Human embryonic kidney 293T cells were grown as specified in Example 3.a. Cells were co-transfected using the Superfect Transfection Kit (Quiagen), with 5 μg total DNA of the following effectors: pCDNA/Lac-z reporter plasmid and one of the following:

1). Expression plasmid encoding RY peptide.

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2). Expression plasmid encoding Bax.

3). Expression plasmids, encoding Bax and RY peptide.

After 18 hours, the cells were harvested for beta-galactosidase activity assay.

Results: As shown in Figure 7, the RY peptide was markedly active in counteracting Bax toxicity.

Conclusion: RY peptide is a potent inhibitor of Bax cell-killing effect in this experimental system.

Example 7: RY peptide inhibits hyfrogen peroxide toxicity in Hela cells

The objective of this experiment was to examine the ability of RY peptide to inhibit cell death induced by hydrogen peroxide in HeLa cells.

Methods: Two methods for administration of RY peptide were employed:

1) Hela cells were co-transfected (via electroporation), with two expression vectors: (1) Expression vector encoding for the reporter gene beta-galactosidase. (2). Expression vector encoding for RY peptide (as described in Example 2).

2) Hela cells were co-transfected (via electroporation), with the expression vector encoding for beta-galactosidase and with the synthetic RY peptide (as described in Example 1).

Five hours after the electroporation procedure, cells were tested with 100μM and 200μM of hydrogen peroxide (Merck, Inc. USA), for 18 hours.

As described in Figures 8A and 8B, hydrogen peroxide treatment reduced cell survival rate to 20.4±2.8 and 5.8±2.1 percent of control (mean ± SD), respectively. RY peptide, both plasmid-encoded and synthetic peptide, manifested a marked protective effect. Cell survival rate in the presence of hydrogen peroxide and plasmid-encoded RY peptide was increased to 53.9±9.3 and 15.3±4.2 percent of control, respectively. Protective effect of synthetic RY peptide was reflected by increase of cell survival rate to 53.3±8.8 and 33.4±3.7%, respectively ($p<0.001$).

Figure Legends:

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Figure 1: Structure of the RY region

NMR solution three-dimensional structure of Bcl-xL (Muchmore SW, et al., Nature 381: 335-341, 1996) was extracted from the Brookhaven Protein Data Bank, Accession No. 1LXL. The RY region (Phe97-Ph105), is marked. As shown, this small region is located on the outer surface of the protein, forming an almost closed loop.

Figure 2: RP-HPCL of the purified RY peptide.

RP-HPLC (Reversed phase high performance liquid chromatography) of purified RY peptide (H-Phe-Glu-Leu-Arg- Tyr-Arg- Arg-Ala-Phe-OH). The peptide was analysed on C_{18}-5 μm Phenomenex Kromasil column (4.6 mm. I.D. X 25 cm). A sample was eluted using the following gradient: A, distilled H_2O 0.05% TFA (Trifluoroacetic acid)/;Acetonitrile 0.05% TFA; λ=214; flow 1 ml/min. t=0%B=5 t=30min %B=50. The numbers on the y axis indicate mili absorbance units. The numbers on the X axis indicate minutes. The numbers on the peaks of the graph indicate the retention time.

Figure 3: MS-(FAB) of the purified RY peptide

MS-(FAB) (Mass spectra-Fast atomic bombardment) of purified H-Phe-Glu-Leu-Arg-Tyr-Arg-Arg-Ala-Phe-OH. The numbers on the y axis indicate the relative ion abundance of the ms detector: On the x axis the mass per charge M/z is calculated for $C_{59}H_{89}N_{18}O_{13}$ (MH^+) 1257.7, and the M/z found was 1258.4.

Figure 4: In vitro assay system for the Bcl-2 family of proteins

HeLa cells were co-transfected with a beta-galactosidase reporter plasmid in combination with either a control plasmid (vector), a plasmid expressing Bcl-2 or Bax, or both plasmids expressing Bcl-2 and Bax, using electroporation procedure. Cell viability was assessed at 18 hours past the electroperotion, by measurement of beta-galactosidase activity.

Figure 5: RY peptide protects HeLa cells against dopamine toxicity:

Synthetic RY peptide was introduced into HeLa cells with a

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beta-galactosidase reporter plasmid by electroporation. After five hours, cells were exposed to dopamine (150μu and 300μM, Sigma, Ltd., Israel) for 18 hours. Cell viability was then evaluated by measurement of the beta-galactosidase activity.

Figure 6: RY peptide increases cell survival rate and inhibits Bax-induced cell death, thus mimicking the effect of Bcl-2.

HeLa cells were co-transfected by electroporation with an expression plasmid encoding the RY peptide and a beta-galactosidase reporter plasmid, and introduced into the assessment system of the Bcl-2 proteins, as described above in Example 3.b.3 and Figure 4. Cell viability was evaluated 18 hours after the electroporation by measurement of beta-galactosidase activity.

Figure 7: RY peptide inhibits cell-killing effect of Bax on embryonic kidney 293T cells

Human embryonic kidney 293T cells were co-transfected by electroporation with a reporter plasmid encoding beta-galactosidase, in combination with either an expression plasmid encoding RY peptide; an expression plasmid encoding Bax; or expression plasmids encoding Bax and RY peptide. Cell viability was evaluated after 18 hours, by measurement of beta-galactosidase activity.

Figure 8A: Plasmid-encoded RY peptide protects against hydrogen peroxide toxicity.

HeLa cells were co-transfected via electroporation with an expression plasmid encoding for RY peptide, together with a reporter plasmid encoding for beta-galactosidase. Five hours after electroporation, cells were treated with either 100μM or 200μM of hydrogen peroxide (HP) for 18 hours. Cell viability was then evaluated by determining beta-galactosidase activity.

Figure 8B: Synthetic RY peptide protects against hydrogen peroxide toxicity.

HeLa cells were co-transfected via electroporation with RY

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Claims:

1. An isolated and purified peptide of the RY domain having an amino acid sequence of general formula I comprising a sequence of the following amino acids:

$$X_1 - X_2 - X_3 - X_4 - X_1 - X_4 - X_4 - X_3 - X_1 \qquad I$$

X_1 = Phe, Tyr, or any amino acid having a substituted aromatic residue;

X_2 = Glu, Asp, Ser, or any amino acid having a $-(CH_2)_n-COO^-$ residue, wherein n = 0-3;

X_3 = Asp, Thr, any aliphatic amino acid, or any of amino acids X_4; and

X_4 = Arg, Lys, or any amino acid having a $-(CH_2)_n-NH_3^+$ residue, or a $-(CH_2)_n-NH-C(NH_3^+)NH_2$ residue wherein n = 0-4;

as well as functional equivalents thereof.

2. A peptide according to Claim 1, wherein Methionine (Met) is connected to the N-terminal of the sequence of general formula I.
3. A peptide according to Claim 1 or 2 wherein in the sequence of general formula I, the sequence is
 $X_4 - X_1 - X_4 - X_4$ stands for Arg - Tyr - Arg - Arg.
4. A peptide according to Claim 3, wherein the sequence is preceded by X_3 = Arg.
5. A peptide according to any of Claims 1 to 4, wherein the substituted aromatic residue of X_1 is Phenyl-$(CH_2)_n$-.
6. A peptide according to any of Claims 1 to 5, wherein the aliphatic amino acid of X_3 is selected among Leu, Ile, Ala, Gly and Val.
7. Phe-Glu-Leu-Arg-Tyr-Arg-Arg-Ala-Phe.
8. Phe-Ser-Arg-Arg-Tyr-Arg-Arg-Asp-Phe.
9. Phe-Glu-Thr-Arg-Phe-Arg-Arg-Thr-Phe.
10. A pharmaceutical composition comprising as active ingredient a RY peptide according to any of Claims 1 to 9 or a functional equivalent thereof.
11. A pharmaceutical composition according to Claim 10 comprising a pharmaceutical acceptable carrier.
12. The use of the RY peptide or of a pharmaceutical composition according to any of Claims 1 to 11 in the preparation of a

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medicament.

13. A method for the treatment of disorders of inappropriate activation of apotosis by a RY peptide or by a pharmaceutical composition according to any of Claims 1 to 11.

14. A method for increasing the number of viable cells in a biological tissue by a RY-peptide or by a pharmaceutical composition according to any of Claims 1 to 11.

15. A method for the enhancement for the survival of biological cells by a RY-peptide or by a pharmaceutical composition according to any of Claims 1 to 11.

16. A method for the preparation of a RY-peptide of general formula I according to Claim 1, which comprises attaching the corresponding amino acids, one after the other, onto a functionalized resin, by the following steps:

a. sythesising the sequence of Fmoc (9-fluorenyl methoxycarbonyl)-N^{alpha}-protected amino acids activated in situ in a suitable synthesizer and coupling same to a preloaded resin, removing the protecting group and repeating the coupling and deprotecting steps until the entire peptide synthesis has been finalized;

b. cleaving the peptide from the resin,; and

c. purifying the peptide obtained in step b.

17. A method according to Claim 16, wherein the synthesizing step is performed by using an ABI (Applied Biosystems U.K.) 433 A synthesizer.

18. A method according to Claim 16 or 17, wherein the coupling reagent is HBTU/HOBt (benzotriazole-N,N,N',N'-tetramethyl-uronium hexafuorophosphate/N-hydroxybenzotriazole).

19. A method according to any of Claims 16 to 18, wherein 3 equivalents of each of the activated amino acids is used in each coupling step.

20. A method according to any of Claims 16 to 19, wherein the resin is selected among a Wang resin and a 2-chlorotrityl resin.

21. An isolated and purified peptide of the RY domain having an amino acid sequence of general formula I as defined in Claim 1 and 2, substantially as herein described with reference to the examples.

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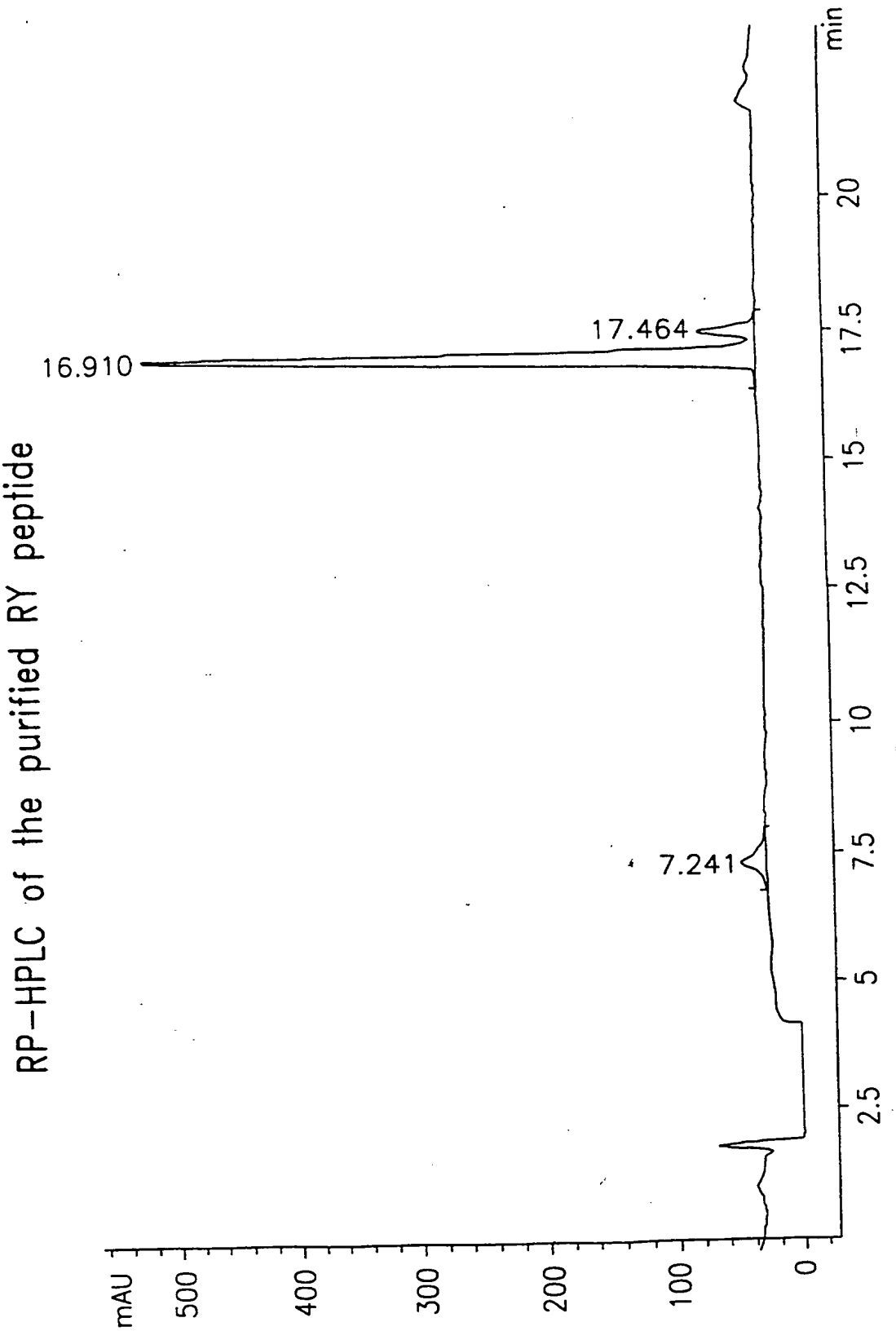

RP-HPLC of the purified RY peptide
16.910
17.464
7.241
mAU
500
400
300
200
100
0
min
2.5
5
7.5
10
12.5
15
17.5
20

FIG.2

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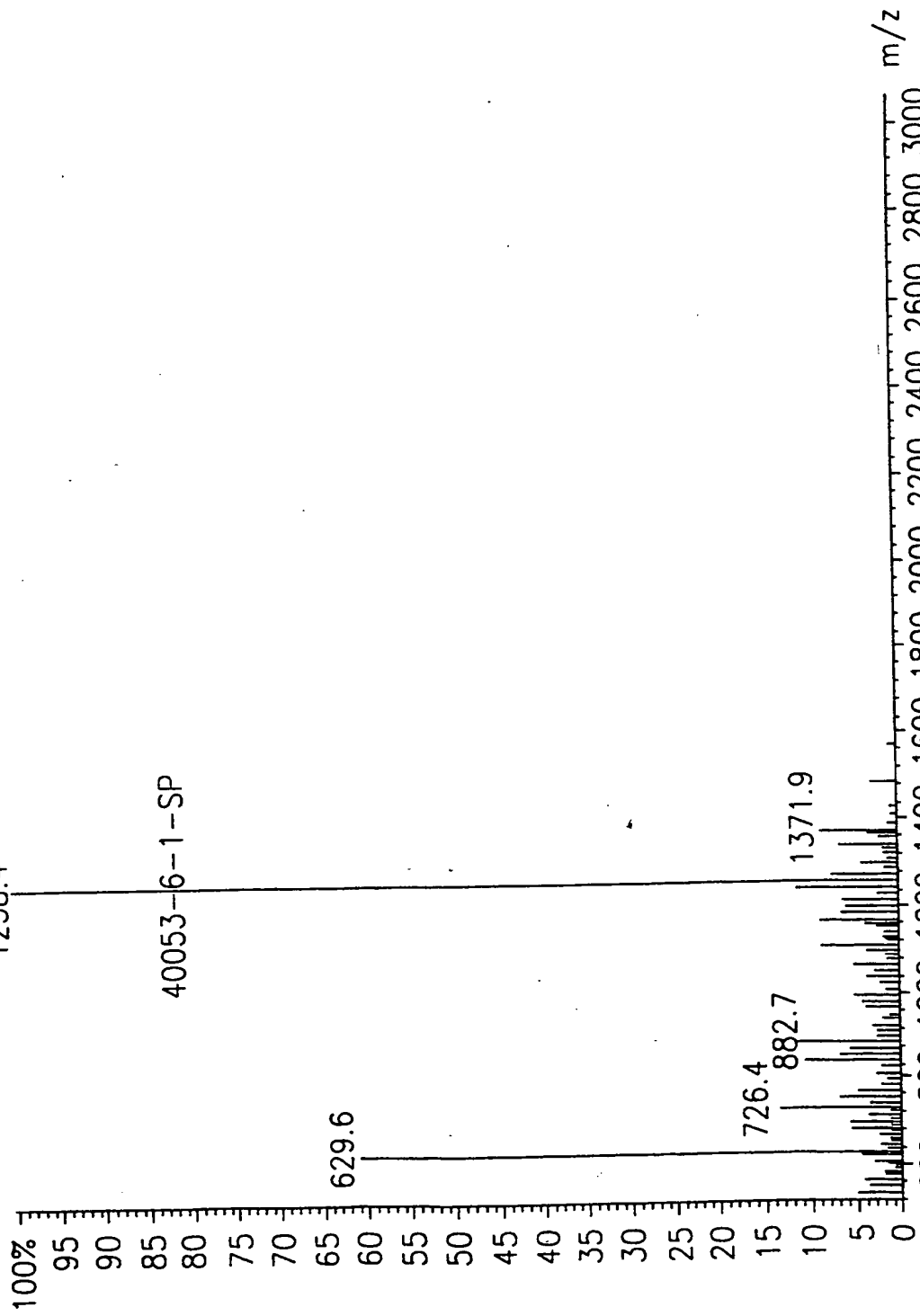

MS-(FAB) of the purified RY Peptide
1258.4
40053-6-1-SP
1371.9
882.7
726.4
629.6
100%
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600
2800
3000
m/z

FIG.3

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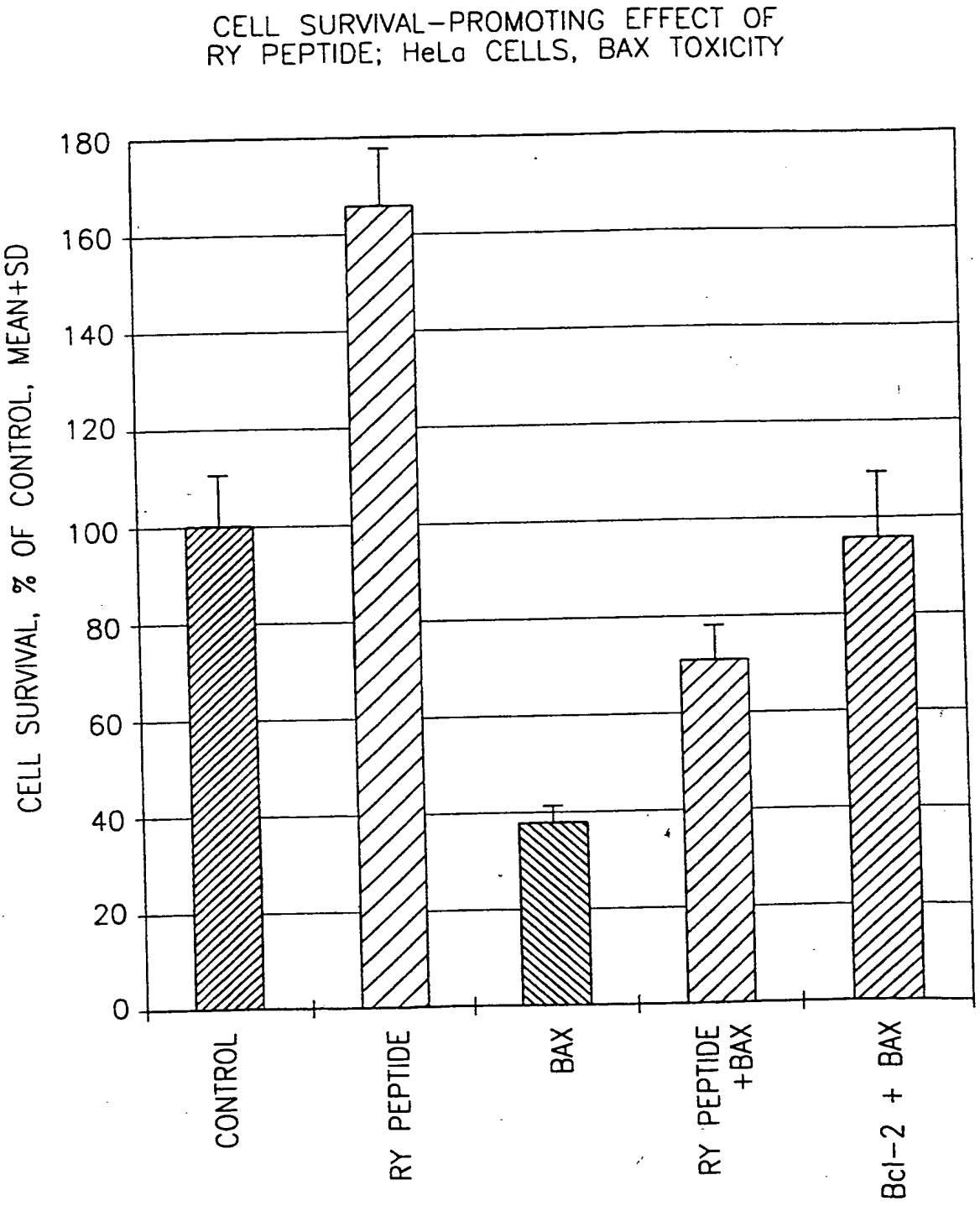

CELL SURVIVAL–PROMOTING EFFECT OF
RY PEPTIDE; HeLa CELLS, BAX TOXICITY
CELL SURVIVAL, % OF CONTROL, MEAN+SD
180
160
140
120
100
80
60
40
20
0
CONTROL
RY PEPTIDE
BAX
RY PEPTIDE +BAX
Bcl-2 + BAX

FIG.6

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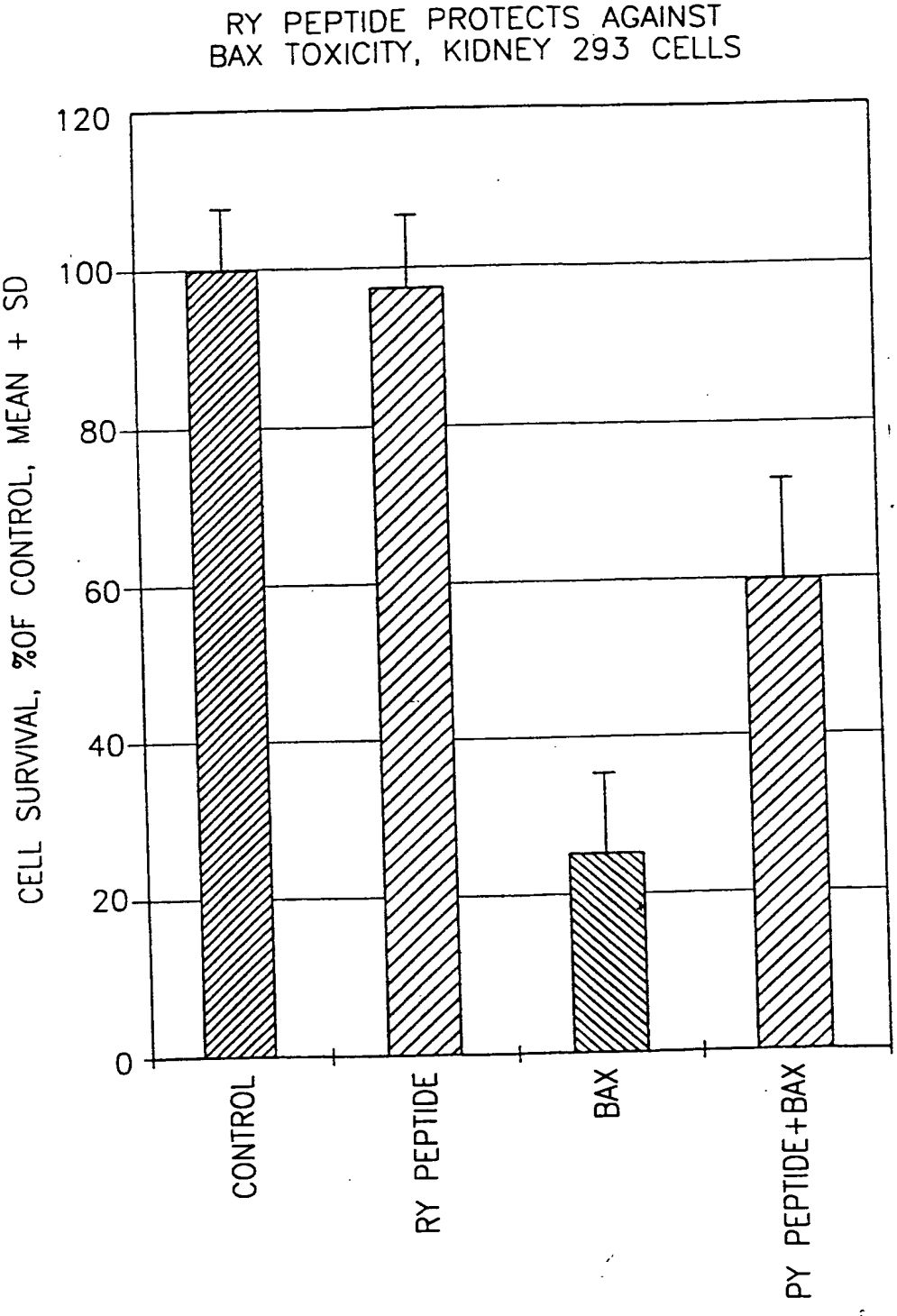

RY PEPTIDE PROTECTS AGAINST
BAX TOXICITY, KIDNEY 293 CELLS
CELL SURVIVAL, %OF CONTROL, MEAN + SD
120
100
80
60
40
20
0
CONTROL
RY PEPTIDE
BAX
PY PEPTIDE+BAX

FIG.7

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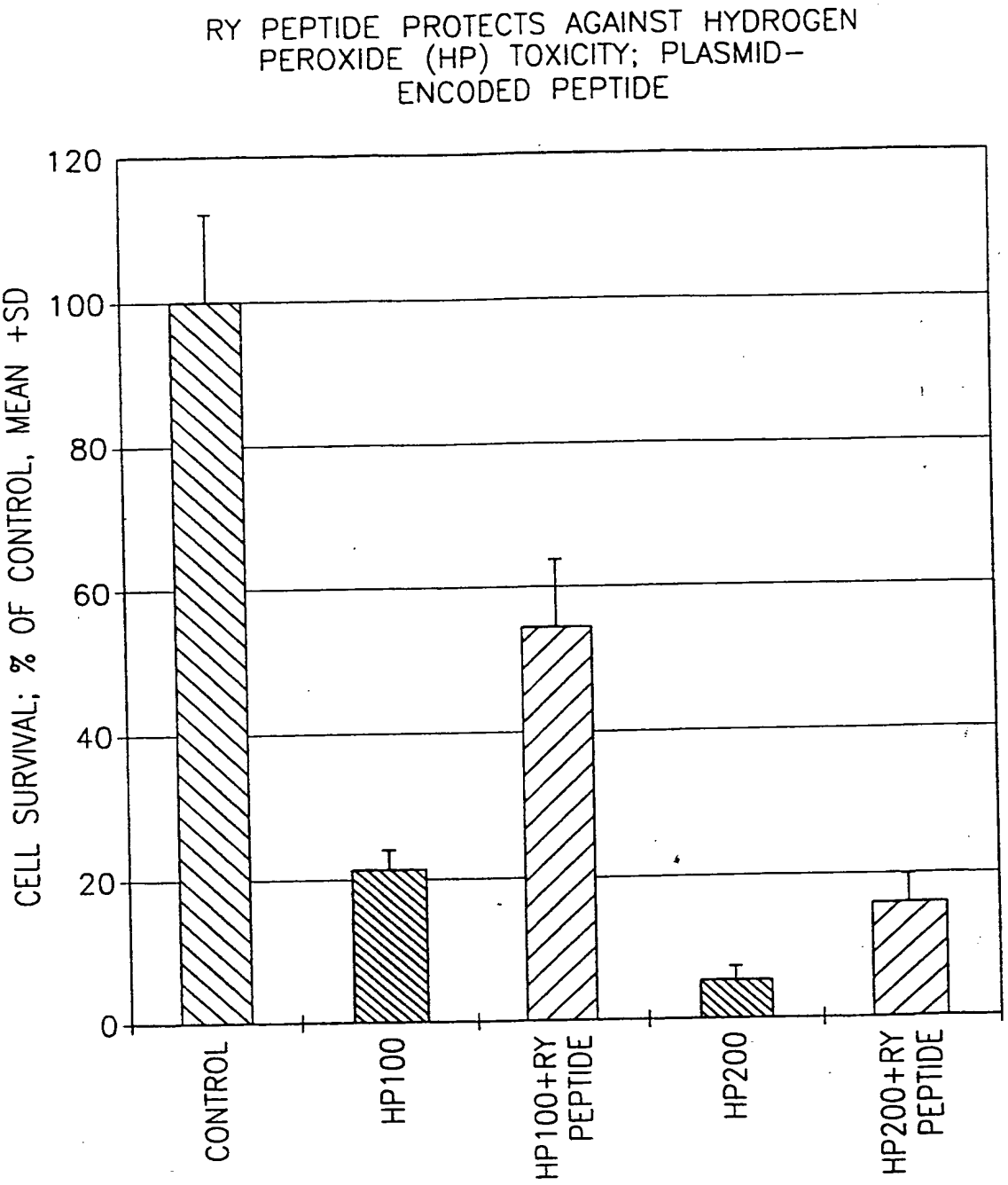

RY PEPTIDE PROTECTS AGAINST HYDROGEN PEROXIDE (HP) TOXICITY; PLASMID-ENCODED PEPTIDE
CELL SURVIVAL; % OF CONTROL, MEAN +SD
120
100
80
60
40
20
0
CONTROL
HP100
HP100+RY PEPTIDE
HP200
HP200+RY PEPTIDE

FIG.8A

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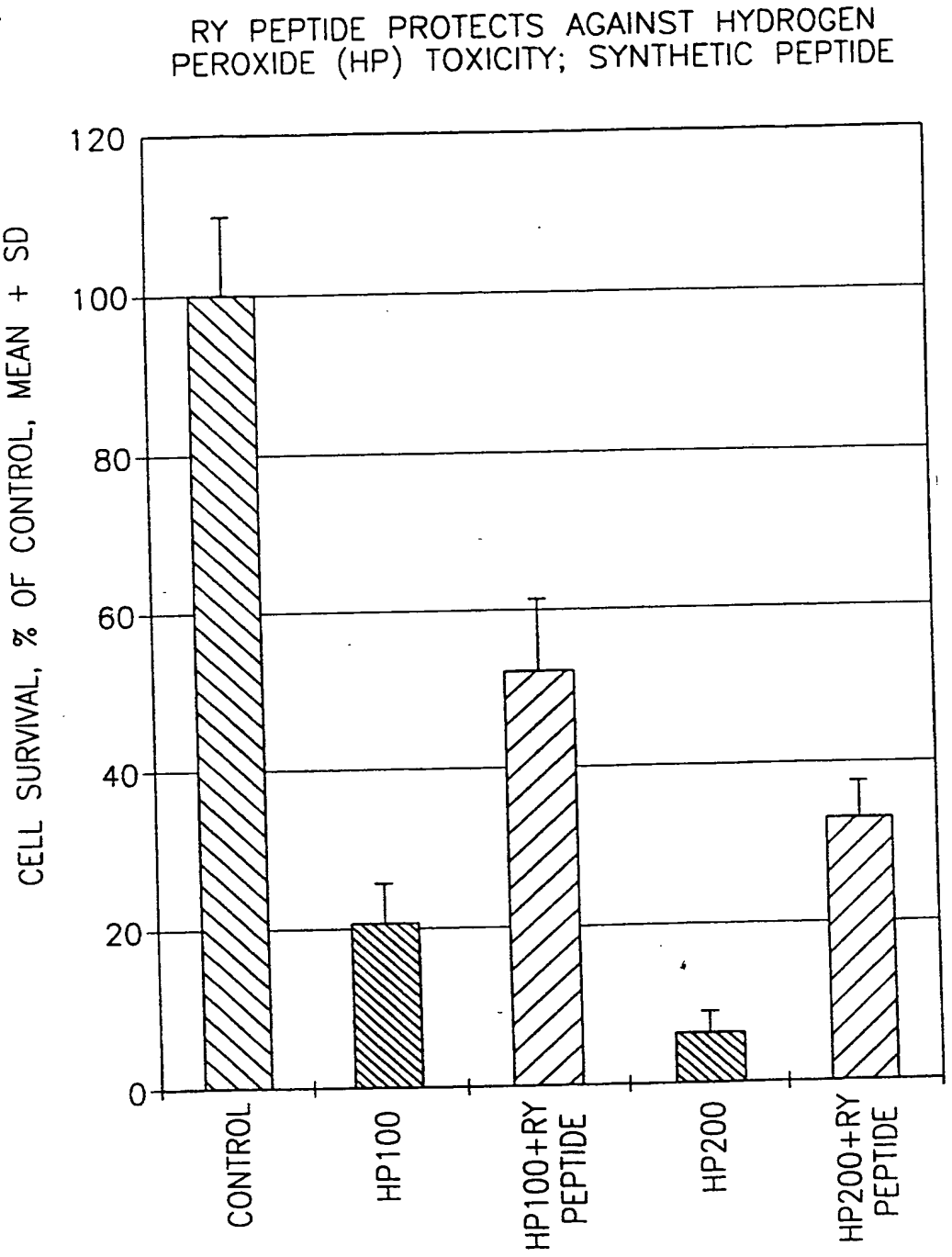

RY PEPTIDE PROTECTS AGAINST HYDROGEN PEROXIDE (HP) TOXICITY; SYNTHETIC PEPTIDE
CELL SURVIVAL, % OF CONTROL, MEAN + SD
120
100
80
60
40
20
0
CONTROL
HP100
HP100+RY PEPTIDE
HP200
HP200+RY PEPTIDE

FIG.8B

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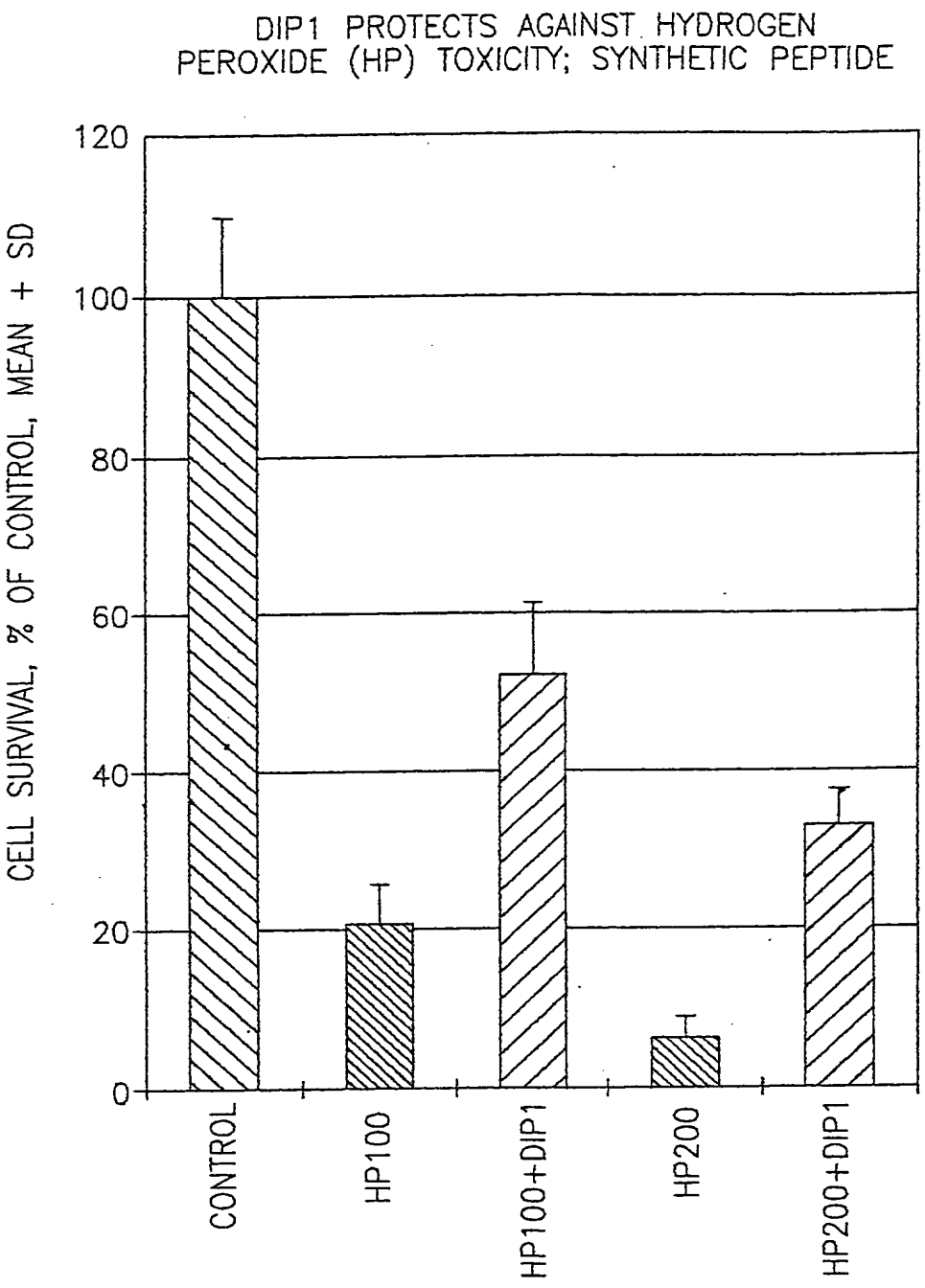


FIG.8B

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